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A COMPARISON OF THE EFFECT OF NINE ANTIBIOTICS ON EXPERIMENTAL TYPHOID INFECTIONS IN MICE

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STANLEY W. WOLFSON, B.S.
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IN EVALUATING drugs, particularly antibiotics, for their usefulness in the treatment of human or animal diseases it is customary to obtain preliminary data based on in vitro tests, and later to employ in vivo tests in animals. If the drug is suitable for human use it is then cautiously used in man for infections caused by microorganisms against which the in vitro or in vivo screening tests have shown it to be active.

In view of the large amount of antibiotic screening work that is now being carried on by various manufacturers and other groups, it seems desirable to point out certain limitations of the usual in vitro and in vivo tests that are used in evaluating new antibiotics or other drugs. Fortunately, by and large, such tests do give a good idea as to the probable value of the drug under test in the treatment of human diseases. The present study indicates, however, that in certain instances one can not rely on such limited tests to determine the usefulness of a drug in man.

The inadequacy of in vitro tests was strikingly demonstrated several years ago when penicillin K was shown to be superior to penicillin G in the test tube[1] but markedly inferior when tested in animals and man. Conversely, penicillin X had an in vitro potency of 900 units per milligram as compared with 1,667 for G and 2,200 for K, but both animal experiments and clinical trial showed that penicillin X was quantitatively more active than penicillin G or K.

Further evidence showing the disparity between test tube and animal experiments has been demonstrated in this laboratory in studies on the synergistic action of antibiotics and sulfonamides on *Salmonella typhosa*. It was reported[2] that under the test conditions only about 50 per cent of the combinations tested showed a positive correlation between in vivo and in vitro assay.

The present work deals with the effect of nine different antibiotics on experimental typhoid infections in mice. In addition to the CD_{50}, the LD_{50} dose of each antibiotic was determined, using mice of the same strain as that used in the protection tests. A therapeutic index could thus be calculated.

EXPERIMENTAL

The antibiotics studied were chloramphenicol, circulin (Q-19), (described by Murray and Tetrault,[3] and Murray and co-workers[4]), polymyxin B, strepto-

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mycin HCl, aureomycin, neomycin, dihydrostreptomycin, penicillin G, and aureotracin (the latter a possible chemical entity formed by the combination of bacitracin and aureomycin).

The antibiotics were diluted on a weight basis with distilled water to the desired concentrations, and 0.5 ml. was injected intraperitoneally into white mice. The mice used weighed between 17 and 25 grams. In the case of chloramphenicol, from 1 to 2 ml. of alcohol were used to solubilize the weighed antibiotic, and additional dilutions were made in distilled water. To prevent crystillization during injection at the higher concentrations (i.e. LD_{50} quantities), the syringe was warmed to about 40 to 50° C. With aureotracin, which is relatively insoluble in quantities above 100 μg per milliliter, the weighed antibiotic was ground in a mortar, suspended in the appropriate volume of distilled water, and rapidly injected.

In determining the CD_{50}, the antibiotics were injected immediately prior to the injection of the test organism (*S. typhosa*, strain 413). The materials were weighed and diluted to the desired concentrations on the same day of testing to assure the efficacy of some of the less stable antibiotics. Since it is an established fact that mucin increases the virulence of most organisms, two challenging doses of *S. typhosa* with and without mucin were used. The injection consisted of 0.6 ml. of a six-hour penicillin assay broth culture (Penassay Broth, Difco) of *S. typhosa* incubated at 37° C. For the mucin experiment, the six-hour broth culture was diluted 1:1,000 in 4 per cent mucin and injected in the same volume.

Forty mice were used for each dilution of antibiotic for the protection test and ten mice for each dilution in the determination of the LD_{50}. The same strain of mice was used throughout these studies. Deaths were recorded after forty-eight hours and semilogarithmic probit paper was used in calculating the CD_{50} and LD_{50}. In each case, death of from 90 to 100 per cent of animals was noted in all control groups. The CD_{50} with and without mucin, the LD_{50}, and the therapeutic indices are shown in Table I.

TABLE I. THERAPEUTIC INDICES OF NINE ANTIBIOTICS IN EXPERIMENTAL SALMONELLA TYPHOSA INFECTIONS IN WHITE MICE

ANTIBIOTIC USED	I.P. CD_{50} (μG/MOUSE)		I.P. LD_{50} (μG/MOUSE)	THERAPEUTIC INDEX	
	NO MUCIN	MUCIN		NO MUCIN	MUCIN
Streptomycin HCl	2.8	12	6,000	2143	500
Dihydrostreptomycin	9	—	11,000	1220	—
Penicillin G	220	2,000	100,000	454	50
Aureotracin	140	—	30,000	214	—
Circulin (Q-19)	2.5	8	420	164	52
Aureomycin	40	40	5,000	125	125
Polymyxin B	2.5	5	260	104	52
Neomycin	75	—	4,000	53	—
Chloramphenicol	1,000	1,200	9,600	10	8

Upon examining the efficacy of these antibiotics on the basis of their CD_{50} alone, it can be seen that the polypeptides (circulin [Q-19] and polymyxin) are the most effective in these experimental infections. It should be noted how-

ever that polymyxin is toxic when administered parenterally in man. At this time the toxicity of circulin for man has not been determined. In general, the polypeptide antibiotics do not appear to be promising because they are not absorbed to any appreciable extent from the gastrointestinal tract and their inherent toxicity precludes their usefulness if given parenterally. Next to circulin and polymyxin B, the most effective antibiotic is streptomycin. It can also be seen that this antibiotic has the highest therapeutic index. As might be expected, dihydrostreptomycin is the next most efficacious drug, followed by aureomycin, neomycin, aureotracin, penicillin, and, last, chloramphenicol. The least effective drug under these experimental conditions, chloramphenicol, also has the lowest therapeutic index. Contrary to these results, however, clinically it is far superior to any of the other antibiotics which have been used in the treatment of typhoid fever in man.

This anomalous behavior of chloramphenicol has been noted elsewhere. Bliss and Todd[5] reported that chloramphenicol was active against streptococcal and pnuemococcal infections in vitro but failed to protect mice in vivo. Conversely, Robinson and associates[6] have shown that although chloramphenicol is ineffective against *Treponema pallidum* in vitro, it is effective in healing the lesions of primary and secondary syphilis.

Additional experiments were carried out in which the infecting dose of culture diluted 1:100 in 4 per cent mucin was given four hours previous to the CD_{50} dose of antibiotic. These results are given in Table II.

TABLE II. TIME STUDY

ANTIBIOTIC ($1 \times CD_{50}$)	PER CENT PROTECTION
Penicillin	82.5
Circulin (Q-19)	72.5
Chloramphenicol	65.0
Streptomycin	27.5
Aureomycin	22.5
Polymyxin B	7.5

In each case a 50 per cent survival rate could be expected if there was no alteration in the effectiveness of the antibiotic injected four hours after the culture. Three antibiotics, penicillin, circulin, and chloramphenicol, gained from 15 to 32.5 per cent in protection. It can be seen that by this technique the order of effectiveness is entirely different from that found in the tests in which the infective dose and the antibiotic were administered at the same time. Thus, by a slight alteration of technique, varying results have been obtained.

DISCUSSION

The results of this investigation indicate that one should not assign too much importance to laboratory data on the efficacy of a drug, particularly when only one species of animal is employed. Conclusions as to the effectiveness of a given drug or drug combination against an organism cannot be drawn on the basis of either in vivo or in vitro laboratory tests. These results emphasize again the necessity for conclusive data by clinical trial in man. In vitro tests can

demonstrate only the activity of a drug or the lack of it in the test tube. It tells little or nothing about the effectiveness of the drug in the animal body. It is of interest to note how well this fact applies to a more recent development in the field of drugs. Recently a thiosemicarbizone (TB1), described by Domagk,[7] has been reported by Sturm[8] to be clinically effective in the treatment of tuberculosis. However, in vitro experiments carried out in this laboratory on the drug recrystallized from commercial tablets have failed to prove its effectiveness on saprophytic acid-fast organisms (*ranae*, *phlei*, *avium*, and *smegmatis*).

Although animal data are of value in giving a broad picture of the toxicity and efficacy of a drug, when such data are favorable it is not possible to assume that similar favorable data will be obtained when the drug is used in man. However, one would hesitate to attempt trial of a drug in man without first testing it in animals. It is essential that such animal tests be exhaustive since one may get an entirely erroneous impression from the study of a drug in a single species. It would appear that in vivo studies of a drug to determine its safety and efficacy should include the use of several species of animals, since, although proper evaluation of a drug must rely in the last analysis on clinical evidence, it is essential that preliminary screening studies be made in lower animals.

CONCLUSIONS

The effects of nine antibiotics on experimental typhoid infections in mice were studied. The LD_{50} and CD_{50} were established for each antibiotic and from these data the therapeutic indices were calculated.

The results obtained were in contrast to the reported clinical results of the therapy of typhoid fever in man.

A slight alteration in the technique of the testing procedure has given a totally different evaluation of the order of antibiotic effectiveness.

These tests demonstrate the fallacy of accepting the efficacy of certain antibiotics solely on the data presented from a limited type of in vivo animal test.

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PLASMA VISCOSITY

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THE purpose of this study was to investigate the values of plasma viscosity in apparently healthy adults and those known to be ill with known disease processes.

MATERIALS AND METHODS

This study was executed in a manner similar to that previously followed for the determination of the blood sedimentation rate in healthy girls.[1]

The patients examined were adults and residents of the state of Vermont. Each individual was given the usual physical and laboratory examinations which included, routinely, serologic tests for syphilis, undulant fever, typhoid fever, paratyphoid fever, a tuberculin test, chest x-ray film with serial retakes when needed, and blood morphology examinations as indicated.

A definite attempt was made to rule out any hidden chronic infection. All apparently healthy individuals were followed through their family physicians, and those with disease processes appearing subsequently that may have been present at the time of the original examination were removed from this group.

At the conclusion of this study there were 361 presumably healthy individuals, and 294 with known disease processes. In the latter group there were 128 patients ill with active pulmonary tuberculosis. Of these, 106 were followed four months and longer. Regular monthly recheck examinations for six months and more were possible in twenty-six persons ill with other diseases.

Potassium oxalate was used as the anticoagulant. All the blood specimens were withdrawn between 9:00 and 11:00 A.M. The sedimentation rates were determined at fifteen-, thirty-, and sixty-minute intervals before noon of the day of examination. The tubes were then centrifuged and the plasma viscosity determinations were made that afternoon. All the tubes were numbered. The sedimentation rates were determined by one technician, and the viscosity values were determined and recorded by another technician.

The earlier viscosity determinations were made by using a pair of identical glass tubes consisting of equal flow capillaries sealed between a larger collecting tube and a measuring tube with a scale for measuring the distance of flow, similar to the Hess viscometer. One tube contained distilled water and the other tube contained the plasma sample (Fig. 1). Both tubes were leveled before each set of determinations. The leveling is necessary to keep the fluids from creeping during a viscosity determination. Both tubes were subjected to the same flow pressure by suction for exactly the same time period. The distance traveled by the plasma was recorded relative to the distance traveled by the water under similar conditions in the capillary of volume, pressure, and time. Both tubes were subjected to as strong simultaneous suction as possible because plasma has different flow characteristics from water, and the viscosity values tend to be high with slow flow and reach a constant value when the rate of flow is highest.

Later viscosity determinations were obtained with the Hellige viscometer. The two tubes used were calibrated one against the other, the volume scale being engraved so as to indicate directly the relative viscosity of the sample liquid (plasma) as compared with the reference liquid (water). If one of the viscometer tubes was broken, they both had to be replaced.

From the Vermont Sanatorium and the Division of Preventive Medicine and Public Health, University of Vermont, College of Medicine.

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The absolute viscosity was obtained by transposing the relative viscosity of water in centipoises for the temperature period at the time the flow distance was noted. At 20° C. the absolute viscosity equaled the relative viscosity. The absolute viscosity for other temperatures was obtained by multiplying the viscometer reading by the coefficient for that temperature:

20° C. — 1.00	23° C. — 0.94
21° C. — 0.98	24° C. — 0.91
22° C. — 0.96	25° C. — 0.89

Examples: If the water moved 1.5 on the scale and the temperature was 20° C., the absolute viscosity of the plasma sample was $1.5 \times 1.00 = 1.5$. If the temperature reached 25° C., the absolute viscosity was $1.5 \times 0.89 = 1.3$.

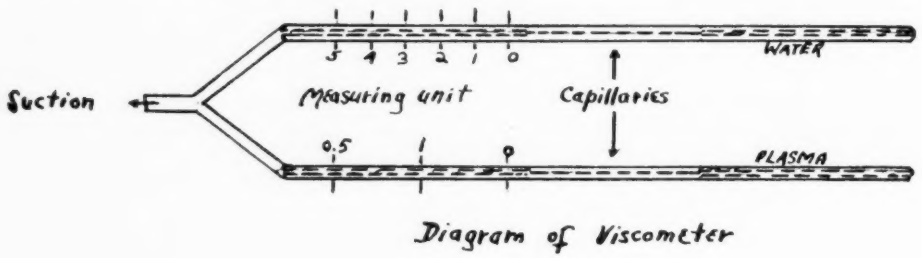

Fig. 1.

With the Hellige viscometer the readings could be interpolated to the second decimal. For practical purposes the second decimal was dropped for all readings except those between 1.6 and 1.7, the "suspicious" zone. If the plasma viscosity appeared to be less than 1.65 and more than 1.60, it was recorded as 1.6+. If it was above 1.65, it was called 1.7.

RESULTS

Simultaneous plasma viscosity and blood sedimentation rates were determined for 361 apparently healthy individuals (Table I).

TABLE I. COMPARISON OF PLASMA VISCOSITY AND BLOOD SEDIMENTATION RATES OF 361 APPARENTLY HEALTHY ADULTS

	PLASMA VISCOSITY		
SEDIMENTATION RATE	1.2 TO 1.6 (NORMAL)	1.6+ TO 2.2 (ABNORMAL)	TOTAL
Less than 12 mm./hr. (normal limits)	81%	5%	86%
More than 12 mm./hr.	13%	1%	14%
Total	94%	6%	

As noted, the plasma viscosity remained 1.6 and less in 94 per cent of the individuals considered to be without evidence of inflammatory, degenerative, or hyperplastic disease. Approximately 6 per cent gave readings above 1.6. The plasma viscosity values did not exceed 1.6+ in any of this group.

The sedimentation rate was within the abnormal zone in 14 per cent of these presumably healthy individuals.

Similar simultaneous studies of plasma viscosity and blood sedimentation rates in 294 adults with known disease processes are noted in Table II. These individuals were ill with the following diverse inflammatory diseases: pulmonary tuberculosis (128 cases), acute and chronic pneumonitis, chronic

bronchitis with bronchiectasis, silicosis, cancer of the lung, pulmonary infarction, sarcoidosis, acute and subacute sinusitis, brain abscess, subacute bacterial endocarditis, carcinoma of the stomach, concealed carcinoma of the intestine, chronic cholecystitis, intestinal tuberculosis, cystitis, pyelitis, pyelonephritis, endometritis, infectious arthritis, rheumatic fever, tuberculosis of spine and knee, cold abscess, phlebitis of legs, chronic prostatitis, pernicious anemia, myelogenous leucemia, and Hodgkin's disease.

TABLE II. COMPARISON OF PLASMA VISCOSITY AND BLOOD SEDIMENTATION RATES OF 294 ADULTS ILL WITH KNOWN DISEASE

SEDIMENTATION RATE		PLASMA VISCOSITY	
Less than 12 mm./hr. (normal limits)	27%	1.2 to 1.6 (normal limits)	8%
More than 12 mm./hr. (abnormal limits)	73%	1.6+ to 2.2 (abnormal limits)	92%

Comparison of the values of plasma viscosity and blood sedimentation values of adults ill with inflammatory, degenerative, and hyperplastic diseases revealed a correlation of 92 per cent with plasma viscosity values, and 73 per cent with sedimentation rates.

Serial studies of plasma viscosity in apparently healthy individuals revealed no appreciable deviation from the normal limit of 1.6 or less during observation periods of six months. As noted in Table III, the plasma viscosity values remained, on the average, within narrow limits.

TABLE III. SERIAL STUDY OF PLASMA VISCOSITY (V) AND SEDIMENTATION RATES (S) IN 6 APPARENTLY HEALTHY ADULTS

	PATIENT		OBSERVATION PERIOD (MO.)						
	SEX	AGE	1	2	3	4	5	6	CLINICAL CONCLUSIONS
s	F	26	6	7	8	10	4	6	No disease
v			1.5	1.5	1.5	1.5	1.4	1.4	
s	F	25	8	5	9	7	4	4	No disease
v			1.6	1.5	1.6	1.5	1.5	1.5	
s	F	22	13	10	9	8	9	5	No disease
v			1.6	1.6	1.6	1.5	1.6	1.4	
s	F	42	10	13	15	14	13	10	No disease
v			1.6	1.5	1.6	1.6	1.5	1.4	
s	M	42	11	11	19	13	14	15	No disease
v			1.6	1.6	1.6	1.5	1.5	1.5	
s	F	36	12	12	12	13	12	12	No disease
v			1.5	1.6	1.6	1.6	1.6	1.6	

Menstruation and normal pregnancy appeared to have no influence on plasma viscosity values. Serial study of eight apparently healthy pregnant women showed plasma viscosity values similar to those noted in Table III. Complicating infection appeared to affect the plasma viscosity as in nonpregnant women. An example is given in Table IV.

Rises to abnormal values were noted in persons under observation when inflammatory processes were found to be present. The plasma viscosity values

TABLE IV. FEMALE SUBJECT, AGED 32 YR.: PREGNANCY COMPLICATED BY ACTIVE PULMONARY TUBERCULOSIS FOLLOWED BY APPARENT ARREST AND POST-PARTUM REACTIVATION

	OBSERVATION PERIOD (MO.)				
	3	4	5	6	10
Sedimentation rate					
mm. in 15 min.	12	8	17	10	6
mm. in 30 min.	21	14	23	20	12
mm. in 60 min.	29	28	27	25	20
Plasma viscosity	1.7	1.5	1.6	1.5	1.8
Pregnancy (mo.)	5	6	7	8	(3 mo. post-partum)
Clinical condition	Active	Improved		App. arrest	Reactivation

reached normal levels following the disappearance of clinical evidence of inflammation (Table V).

Patients ill with pulmonary tuberculosis generally showed plasma viscosity values which were proportional to the degree of clinical evidence of ac-

TABLE V. SERIAL STUDY OF PLASMA VISCOSITY OF 10 PERSONS ILL WITH ACUTE PNEUMONITIS DURING PERIOD OF OBSERVATION

MONTH OF ILLNESS	OBSERVATION PERIOD (MO.)						PATIENT	
	1	2	3	4	5	6	SEX	AGE
1st	1.9	1.6	1.5	1.5	1.4	1.4	F	26
1st	1.7	1.5	1.5	1.6	1.4	1.5	F	46
1st	1.8	1.6+	1.6	1.5	1.4	1.5	F	37
1st	1.6+	1.5	1.6	1.6	1.5	1.4	F	20
1st	1.7	1.5	1.6	1.6	1.6	1.4	F	38
1st and 3rd	1.7	1.5	1.7	1.5	1.5	1.5	F	22
2nd	1.3	1.7	1.6	1.5	1.5	1.4	F	33
2nd	1.5	1.7	1.5	1.6	1.6	1.6	F	34
1st to 4th	2.0	1.8	2.0	1.9	1.6	1.7	M	31
4th	1.5	1.5	1.5	1.6+	1.5	1.5	F	23

tivity. As the tuberculous process regressed or attained stability the plasma viscosity values approached normal limits (Table VI). On the other hand, as the disease progressed and became worse the viscosity values remained within abnormal limits (Table VII).

TABLE VI. SERIAL STUDY OF PLASMA VISCOSITY OF 17 PATIENTS ILL WITH PULMONARY TUBERCULOSIS WHO SHOWED CLINICAL IMPROVEMENT DURING PERIOD OF OBSERVATION

MONTH OF IMPROVEMENT	OBSERVATION PERIOD (MO.)						PATIENT	
	1	2	3	4	5	6	SEX	AGE
2nd	1.7	1.5	1.5	1.5	1.5	1.6	F	34
2nd	1.7	1.5	1.5	1.4	1.6	1.5	M	18
2nd	1.9	1.8	1.9	1.7	1.5	1.7	F	21
2nd	2.0	1.6+	1.5	1.6	1.7	1.6	M	20
3rd	1.6+	1.7	1.4	1.5	1.4	1.5	F	17
3rd	1.6+	1.6	1.5	1.5	1.6	1.5	F	18
3rd	2.1	2.0	2.0	1.9	1.9	1.9	M	44
4th	1.6+	1.7	1.7	1.6	1.7	1.6	F	15
4th	1.8	1.9	1.9	1.8	1.7	1.7	M	58
4th	2.1	2.1	2.0	1.8	2.0	1.9	F	17
4th	1.9	1.9	1.8	1.6	1.6	1.5	F	19
4th	1.8	1.8	1.9	1.7	1.7	1.6	M	29
4th	1.7	1.6+	1.7	1.5	1.5	1.5	F	29
5th	1.9	1.7	1.9	2.0	1.8	1.8	M	18
5th	1.6	1.8	1.8	1.5	1.6	1.8	F	42
5th	1.7	1.6	1.7	1.7	1.5	1.6	F	22
6th	1.5	1.6+	1.8	1.7	1.7	1.5	M	47

TABLE VII. SERIAL STUDY OF PLASMA VISCOSITY OF 15 PATIENTS ILL WITH PULMONARY TUBERCULOSIS WHO SHOWED PROGRESSION OF DISEASE

PATIENT		OBSERVATION PERIOD (MO.)					
SEX	AGE	1	2	3	4	5	6
F	35	1.6+	1.7	1.7	1.7	1.7	1.8
F	61	1.6+	1.6+	1.9	1.7	1.5	1.6+
M	52	1.9	1.8	1.8	1.7	1.8	1.9
F	69	1.8	1.9	2.0	1.7	2.1	1.8
M	35	1.9	2.0	1.9	1.8	1.8	1.9
M	17	1.6	1.7	1.8	1.8	1.7	1.7
M	62	1.9	2.1	1.9	2.0	2.0	1.8
M	35	1.8	1.6	1.9	1.8	1.7	1.7
M	29	1.8	1.6	1.8	1.6	1.8	1.7
M	50	1.7	1.6+	1.6	1.7	1.6+	1.8
F	30	1.7	1.7	1.7	1.7	1.9	1.9
F	21	1.7	1.7	1.6	1.6	1.7	1.7
F	16	1.7	1.6	1.8	1.8	1.8	1.9
F	27	2.0	2.0	1.7	2.1	1.6	2.0
F	35	1.7	1.8	1.6+	1.7	1.8	1.8

The patients ill with pulmonary tuberculosis who died during the period of observation had high plasma viscosity values which occasionally approached normal levels a few days to three months before death (Tables VIII and IX).

TABLE VIII. SERIAL STUDIES OF PLASMA VISCOSITY (V) AND SEDIMENTATION RATES (S) OF PATIENTS ILL WITH PULMONARY TUBERCULOSIS WHO DIED

	PATIENT		OBSERVATION PERIOD (MO.)						
	SEX	AGE	1	2	3	4	5	6	CLINICAL CONCLUSION
s	M	59	28	31	32	19			Progression of disease to death
v			1.6	1.9	1.8	1.4			
s	M	52	27	27	31	30			Progression of disease to death
v			2.0	2.3	2.1	2.0			
s	M	76	28	32	27	28			Progression of disease to death
v			1.8	1.8	1.9	2.0			
s	M	37	23	22	25				Progression to death
v			1.7	1.9	2.1				
s	F	36	28	30	31				Rapid spread
v			1.9	1.9	1.9				Progression to death
s	F	35	27	26	27				Progression of disease to death
v			1.9	1.7	2.0				

TABLE IX. FEMALE SUBJECT, AGED 35 YR.: HODGKIN'S DISEASE (LUNG, HILAR, AND RETROPERITONEAL LYMPH NODES) AND MILIARY TUBERCULOSIS (LUNGS, LIVER, AND SPLEEN)—AUTOPSY DIAGNOSIS

	OBSERVATION PERIOD (MO.)					
	1	2	3	4	5	7
Sedimentation rate						
mm. in 15 min.	25	25	32	15	19	(Died)
mm. in 30 min.	30	28	34	28	32	
mm. in 60 min.	32	30	37	30	32	
Plasma viscosity	2.0	2.0	1.7	2.1	1.6+	

DISCUSSION

Viscosity is the measure of that property of any liquid which produces resistance to flow. When capillary tubes are used for determining viscosity the unit of measurement is the centipoise which is directly proportional to the

radius of the capillary, the time of flow, and the pressure across the capillary and is inversely proportional to the length of the capillary and the volume of flow.

In this study the viscosity of plasma was compared with that of distilled water under exactly similar conditions as to velocity of flow, length and diameter of capillary tube, and particular temperature at the time of determination. The absolute viscosity was expressed in centipoises based on the reference liquid water which has its relative viscosity equal to absolute viscosity of 1.00 centipoise at 20° C.

The normal level of whole blood viscosity has been found to be about five times that of water. It has been found to be increased by ingestion of large amounts of protein and fat, blood concentration, ether, morphine, and work. With increase in viscosity there has been noted peripheral resistance, capillary blood flow decrease, and cardiac work increase.

Blood viscosity has been found to be decreased by starvation and venesection. This decrease has been noted to be present when there has been an increase in the albumin-globulin ratios.

Whole blood viscosity appears to be affected to a great extent by the number and size of the red blood cells. The viscosity of the plasma is not changed by this variable and it can be measured, therefore, more accurately.

Plasma viscosity appears to vary only with the shape of the constituent molecules and the degree of hydration. It appears to be independent of the size of the molecules. It measures an average property of all the molecules without distinguishing between the components of the mixture. Long molecules increase blood viscosity and lead to cell agglutination.[2]

The values for plasma viscosity in healthy individuals are not definitely known. A previous study of thirty-seven normal controls showed thirty to have a plasma viscosity less than 1.585 and seven up to 1.626.[3, 4]

Plasma viscosity appears to be a valuable test for the determination of complicating illness during normal pregnancy. The sedimentation rate loses its value as pregnancy progresses. It increases at the fourth month and reaches its maximum at term.[5] This increase of sedimentation rate in pregnancy appears to be due to changes in the plasma.[6] These changes which occur in normal pregnancy do not seem to influence the plasma viscosity values.[7] Plasma viscosity in eclampsia, before the onset of convulsions, has been found to be 1.25 to 1.50, and during the acute stage of eclampsia, 1.7 to 2.3.[8]

During this study the possible relationship between total plasma protein and plasma viscosity was considered. Simultaneous determinations in thirty patients with active pulmonary tuberculosis showed plasma protein values from 6.8 to 8.4 mg. per cent (copper-sulfate method). There did not seem to be any relationship between these values and the respective plasma viscosity values.

High total plasma proteins are sometimes found in diseases where plasma viscosity values are high, as in chronic infections due to tuberculosis and in cancer. But whereas the plasma viscosity values appear almost constantly

high in a disease such as tuberculosis, the total plasma proteins are very frequently found within the normal range of 6 to 8 mg. per cent.

Another interesting question is the relationship between plasma fibrinogen content and plasma viscosity. Plasma fibrinogen is increased in cases of acute tissue destruction, and in most infectious diseases. It is also increased during pregnancy and menstruation. Our observations have not demonstrated any increase in plasma viscosity during pregnancy or menstruation. It does not seem possible that the increase in plasma fibrinogen in disease contributes to any extent to the rise of the plasma viscosity.

Another important portion of the plasma protein to be considered is albumin. An increase in the albumin fraction is extremely rare. A decrease in albumin is seen in nephritis with lasting albuminuria, and in diseases with marked ascites and edema formation. Advanced tuberculosis shows a decrease in albumin quite frequently. Consequently, albumin cannot be considered a factor contributing to the rise in plasma viscosity so constantly seen in this disease.

Salt fractionation studies of plasma proteins in tuberculosis have shown quantitative globulin fraction increases in active disease.[9] Electrophoretic studies have also shown these changes in the various globulin fractions.[10] Rises in the alpha and gamma globulins with decreases in albumin have been noted in progressive tuberculosis and leprosy. These changes have appeared to parallel the clinical course of the disease. The gamma globulins have shown increases above those values noted in normal subjects; while simultaneous measurement of the total proteins has revealed values low or within normal limits.[10] It is possible that a common agency may produce the rise in plasma viscosity and the change in plasma globulins.

Abnormal viscous proteins and protein-bound serum polysaccharides may play some part in the change in plasma viscosity in some diseases such as multiple myeloma and those concerned with the reticuloendothelial system.

A similar study,[11] utilizing tests commonly employed for this purpose, showed that the zinc sulfate turbidity test was more efficient than the sedimentation rate in separating "disease suspects" by the ratio of 62.6 per cent to 51.5 per cent. Comparing these findings with those of the present study, the plasma viscosity determinations would appear to be highly efficient since the latter exceeded the sedimentation rate in separating "disease suspects" by the ratio of 92 per cent to 73 per cent.

Despite the fact that most of the measurements in this study were made in cases of tuberculosis, plasma viscosity changes are not specific for any single disease process. As noted, plasma viscosity is probably related to some factor or factors in plasma which are present in cases of inflammation, degeneration, and hyperplasia and produce, at the same time, disturbances of normal plasma protein ratios and concentration.

Disease processes which produce such alterations in plasma proteins may be screened by the simple test described for the measurement of plasma viscosity provided the limitations are kept in mind, such as the occasional fall to normal levels in those patients before death.

CONCLUSION

A study is presented of plasma viscosity values in 361 apparently healthy adults and 294 persons ill with known disease.

It is concluded that plasma viscosity offers valuable evidence in clinical medicine as a screening method for the determination of organic disease which may cause physiochemical plasma alterations.

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FACTORS INFLUENCING THE HEAT COAGULABILITY OF SERUM PROTEINS

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DURING an investigation of the enzyme inhibiting action of certain serum proteins[1, 2] it was observed that human serum samples differed considerably in their resistance to coagulation by temperatures above 65° C. The work of Griffin and Baumann[3] suggested that this variation might be of significance in neoplastic disease since they had shown that homogenates of precancerous rat livers failed to coagulate at 100° C. while similar preparations from normal livers solidified within five minutes. In developing methods for preventing heat denaturation of serum albumin intended for use in the tropics, Luck and collaborators[4, 5] found that these preparations could be stabilized by compounds such as sodium caprylate or other substances containing a fairly large nonpolar residue, either aliphatic or aromatic. However, until the report of Huggins and co-workers[6] it was not generally appreciated that similar inhibition of protein coagulation occurs in vivo as a result of endogenous products of disturbed metabolism. An analysis of data obtained in a study of 1,350 patients is presented herein and some of the factors causing prolongation of the heat coagulation time are discussed.

METHOD

Three to 4 c.c. of venous blood are withdrawn into a dry syringe and transferred to a small test tube. After clotting, the serum is separated by centrifugation. 0.30 c.c. of serum is pipetted into a 12 by 75 mm. test tube and placed in a water bath at 70° ± 0.5° C. The number of minutes required for solidification of the serum is recorded as the "heat coagulation time." Normal values range from 3 to 10 minutes, abnormal from 10 to 60 minutes. A few samples fail to coagulate in 60 minutes and are unlikely to do so after longer heating. The test is terminated, therefore, at 60 minutes and any uncoagulated specimen is recorded simply as >60.

RESULTS

Influence of Disease.—Determinations of the heat coagulation time were made on a group of patients routinely admitted to Birmingham Hospital in order to obtain data in a wide variety of unselected pathologic conditions. In addition, the test was performed on 221 healthy subjects who served as controls. Results are summarized in Table I. It will be noted that tests on the majority of healthy persons yielded values of less than 5 minutes and that none had a heat coagulation time greater than 10 minutes. On the other hand, it is apparent that almost

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TABLE I. INFLUENCE OF DISEASE ON HEAT COAGULATION TIME OF SERUM

DIAGNOSIS	TIME IN MINUTES						NUMBER OF CASES	% ABNORMAL (>10)
	<5	5-10	11-15	16-20	21-40	>40		
Healthy controls	125	96	0	0	0	0	221	0
Trauma, surgery	0	0	2	1	12	5	20	100.0
Acute infections	2	14	37	22	32	10	117	86.4
Rheumatoid arthritis	2	16	10	8	6	1	43	58.1
Cancer	13	99	51	31	39	24	257	56.2
Diabetes	1	10	4	5	3	0	23	52.1
Cardiovascular disease	3	42	27	10	7	2	91	50.5
Peptic ulcer	6	52	13	3	1	0	75	36.0
Chronic infections	7	92	34	3	5	3	144	31.2
Benign tumors	2	17	3	0	0	0	22	13.6
Paraplegia, uncomplicated	3	80	3	1	0	0	87	4.5
Unclassified	24	151	44	17	12	6	254	31.1
Total							1,354	

any pathologic state may be associated with diminished coagulability of the serum by heat. Abnormal heat coagulation times were found most commonly in the acutely ill patient either immediately following surgery, injury, or hemorrhage or with acute infection, and less commonly in the more chronic afflictions such as rheumatoid arthritis, cancer, diabetes, heart disease, and tuberculosis. No diagnostic specificity for the procedure could be observed.

Special attention was given to an analysis of the heat coagulation times in cases of neoplastic disease. In this group, all diagnoses were proved by biopsy. Multiple determinations were made, some patients having been followed over six months with fifty or more tests. These data are summarized in Tables II and III. Nearly all patients in the terminal stages of the disease exhibited deficient

TABLE II. SERUM COAGULATION TIMES IN 257 CANCER PATIENTS

	TIME IN MINUTES						
	<5	5-10	11-15	16-20	21-40	>40	TOTALS
Active disease	3	63	48	40	39	24	207
(a) Terminal*	0	5	10	7	20	23	65
(b) Others	3	58	38	23	19	1	142
Clinically arrested	10	36	3	1	0	0	50

*Average of four or more determinations in last month of life.

serum coagulation, but, curiously, 8 per cent failed to show this abnormality. On the other hand, when cancer was known to be present, but was relatively quiescent following radiation, mustard, or hormone therapy, 92 per cent gave normal values. In the entire group of 257 cancer patients there were 44 per cent with heat coagulation times within the normal range.

TABLE III. INCIDENCE OF ABNORMAL HEAT COAGULATION TIMES (OVER 10 MIN.) IN 257 PROVED CANCER CASES

GROUP	NUMBER	PER CENT
Terminal cases	65	92.1
Active cancer, not terminal	142	57.1
Clinically arrested	50	8.0
All groups	257	56.2

Influence of Serum Protein Concentration.—An obvious factor to be considered with respect to heat coagulability of serum is its actual content of total protein. The accompanying scattergraph, Fig. 1, indicates that there is a general tendency for the two to be related, samples with lower protein concentration generally requiring longer to solidify on exposure to heat. This is a possible explanation for the increased heat coagulation times in chronically ill

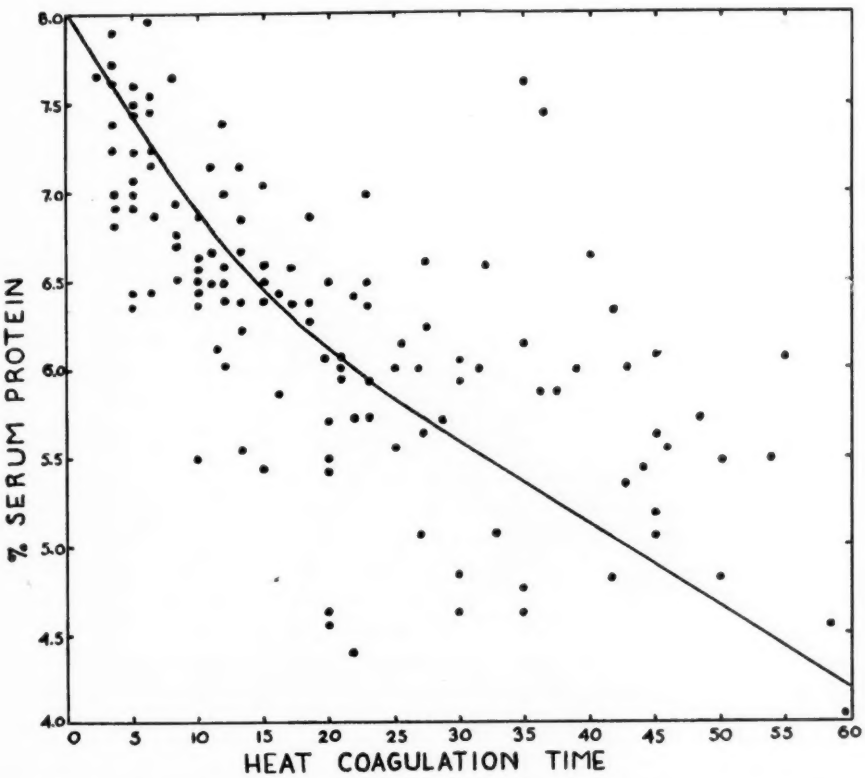

Fig. 1.—Heat coagulation times of serum samples plotted against their total protein concentrations.

patients with hypoproteinemia or in dehydrated patients with hemoconcentration. However, a precise inverse relationship between serum protein concentration and heat coagulation time does not seem to exist. Many exceptions are noted, and it is apparent that additional factors must be concerned in the production of this phenomenon.

Influence of Blood Sugar and Urea Nitrogen.—It has been shown by Ball and associates[7] that various sugars inhibit the heat denaturation of egg albumin. The possible bearing of this finding on the abnormal heat coagulation times observed in the diabetic group was studied by adding glucose to serum in concentrations up to 1,000 mg. per cent. It was concluded that in physiologic amounts blood sugar does not influence the coagulation of serum by heat when measured under these conditions (Table IV). A similar conclusion was

reached in regard to urea nitrogen which was investigated for its possible role in prolonging serum coagulation times in patients having nitrogen retention associated with cardiovascular disorders.

Influence of Adrenal Activity.—Since trauma and surgery were invariably followed by an increase in heat coagulation time of the serum, and since acute infections caused such changes in 86 per cent of patients, the possibility of non-specific stress with activation of the pituitary-adrenal mechanism was considered

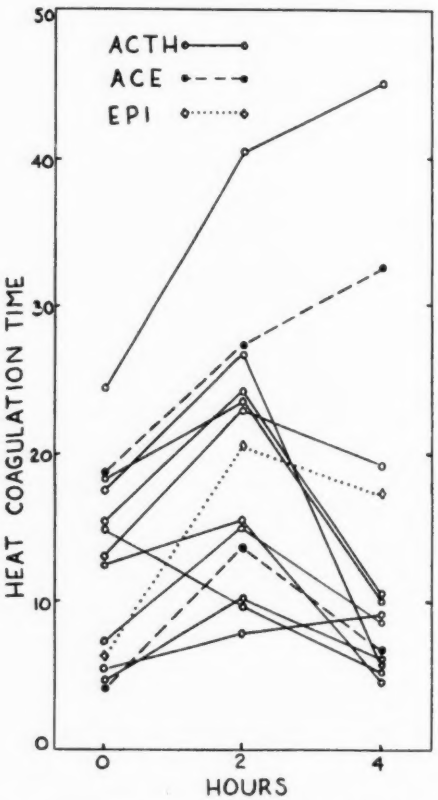

Fig. 2.—Influence of adrenocorticotrophic hormone (*ACTH*), adrenal cortical extract (*ACE*), and epinephrine (*EPI*) on heat coagulation time of serum. Each curve represents one cancer patient.

as a common factor that might be involved. In the course of enzyme studies with adrenocorticotrophic hormone* in cancer patients (unpublished data), the effect of single 25 mg. doses of the drug on heat coagulation of the serum was followed in ten cases (Fig. 2). In the majority a striking increase was found in two hours, with a secondary fall in four hours. Greatest increases were observed where the heat coagulation time was prolonged initially. Similar changes were noted after adrenal stimulation with 1.0 mg. of epinephrine given intravenously in one hour or after intramuscular administration of potent adrenal cortex extract† containing 500 glycogen deposition units in 2 cubic centimeters.

*ACTH (lot H-6009) supplied through the courtesy of Dr. John R. Mote, the Armour Laboratories, Chicago, Ill.

†Adrenal cortex extract kindly furnished by the Upjohn Company, Kalamazoo, Mich.

TABLE IV. INFLUENCE OF ADDED GLUCOSE AND UREA ON HEAT COAGULATION TIME OF A SERUM SAMPLE

GLUCOSE (MG. %)	HEAT COAGULATION TIME	UREA N (MG. %)	HEAT COAGULATION TIME
100	4.0	15	12.0
250	4.0	48	12.0
400	4.0	82	10.5
550	4.5	115	11.0
700	4.0	148	12.5
850	4.0	182	12.0
1,000	4.0	215	12.0

It appears, therefore, that the sudden increase in heat coagulation time of the serum following a variety of nonspecific stresses may be explained in part on the basis of adrenal cortical activation and subsequent metabolic changes. Bennhold[8] has shown that serum albumin has the ability to bind a variety of compounds, at the same time becoming more resistant to heat denaturation. This suggests the possibility that similar binding of substances released into the circulation following adrenal stimulation may account for the rapid increases in heat coagulation time that are observed. Studies are in progress to determine the nature of the factor or factors in the serum which are responsible for this effect.

DISCUSSION

There appears to be no diagnostic significance of the heat coagulation time of serum in relation to cancer or other specific pathologic conditions. Interest in the phenomenon is concerned chiefly with the mechanism by which the defect in protein coagulability appears. While hypoproteinemia is a factor, there is no strict correlation between total serum protein and the heat coagulation time. Furthermore, this does not explain rapid increases in the latter in trauma, surgery, acute infections, or acute phases of chronic disease. These observations suggested that nonspecific stressful stimuli might produce changes in heat coagulation of the serum as an indirect result of adrenal cortical activation. This possibility was confirmed by finding rapid changes in the heat coagulation time after administration of ACTH or adrenal cortical hormone. Although other factors may be involved, it may be suggested that the heat coagulation time is in part an expression of adrenal response to the stress of disease.

SUMMARY

A simple method is described for comparing the resistance of sera to coagulation by heat.

The highest incidence of abnormal values is found in the acutely ill patient and in those recovering from the shock of surgery or injury. The frequency decreases in less overwhelming pathologic states.

Evidence is presented which suggests that adrenal response to the stress of injury or disease may be an important factor influencing the heat coagulation time.

Observations on the heat coagulation time of the serum of 1,350 patients indicate that no diagnostic significance can be ascribed to the procedure but rather that the results reflect a nonspecific disturbance in the metabolism of the diseased patient.

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THE VALUE OF THE HETEROPHILE ANTIBODY REACTION IN THE LYMPHOMATOUS DISEASES

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A POSITIVE heterophile antibody test is now generally associated with infectious mononucleosis in all but rare instances. The specificity of the test has been increased by the use of guinea pig kidney antigen which absorbs and thus eliminates Forssman antibodies. Low titer agglutinations, generally well below diagnostic range, are not infrequently found in nonspecific conditions. In a recent study Schultz[1] reported significant titers in a group of miscellaneous diseases that included the leucemias, lymphomas, and related diseases. As the heterophile agglutination test is often an important aid in differentiating infectious mononucleosis from the diseases mentioned, we are presenting data which have been collected over a two-year period and which we believe will help clarify the interpretation of the reaction in these conditions.

CASE MATERIAL AND METHOD

The case material consists of 458 miscellaneous patients, forty-three patients with infectious mononucleosis, and fifty-five patients with lymphomas and leucemias. Six hundred and thirty-three tests were performed on the sera of the miscellaneous patients, 104 tests on the sera of the patients with infectious mononucleosis, and eighty tests on the sera of the patients with lymphomas and leucemias. Thus, 817 tests were performed on the sera of 556 patients.

A modified Stuart[2] technique is used. Final serum dilutions range from 1:10 through 1:1,280, while the final sheep cell antigen concentration is 0.5 per cent. The tubes are incubated at 37° C. for one hour, then refrigerated overnight. The degree of agglutination is noted after each tube is inverted three times. A test on serum which has been adsorbed with guinea pig kidney antigen is now routinely performed if there is agglutination in dilutions of 1:80 or higher. The patient's inactivated serum is added to guinea pig kidney antigen in a dilution of 1 part of serum to 4 parts of kidney suspension, allowed to stand one hour, and then centrifuged. The supernatant fluid is then subdiluted to the same final dilutions as the unadsorbed serum. The test is performed as before and a simultaneous run of the untreated serum is carried through in parallel so that an accurate comparison of titers can be made. This time, however, all dilutions are incubated at 37° C. for two hours before they are placed in the refrigerator. Agglutination of an unadsorbed serum in a dilution of 1:160 or higher, with an agglutination of adsorbed serum in a dilution of 1:80 or higher, is reported as a positive test.

RESULTS

The sensitivity of the heterophile agglutination reaction as routinely performed is demonstrated by the relationship of positive tests to established cases of infectious mononucleosis. There were forty-three suitable cases in this group. Each case was studied to affirm the fact that either the typical clinical course

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occurred or a characteristic blood smear was present. Only three patients had no agglutination in titers of 1:160 or above; five patients had titers of 1:160; all the others had titers of 1:320 or higher. The adsorption test was performed in twenty-eight cases and caused a fall of more than one dilution in only two cases. In both of these the final titer was still 1:320. These data are summarized in Table I.

TABLE I. HIGHEST TITERS OBTAINED IN 556 PATIENTS
(JULY 1, 1947 TO JUNE 30, 1949)

	TOTAL	NEG.	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280 AND OVER
Infectious mononucleosis	43			1		2	5	4	5	26
Miscellaneous cases	458	412				32	11	2		1
Lymphoma cases	55	51				3	1			
Total cases	556	464				37	17	6	5	27
Infectious mononucleosis after adsorption	28					1	2	2	8	15
Miscellaneous and lymphoma cases after adsorption	25	11	3	5	3	1	1			1
Total cases after adsorption	53	11	3	5	3	2	3	2	8	16

The selectivity of the test was affirmed by reviewing the course of forty-six cases in which a titer of 1:80 or over was found during the two-year period of observation but in which the diagnosis of infectious mononucleosis was not made. There were fourteen instances in which the titer was 1:160 or above. One patient with a titer of 1:2,560 (1:1,280 after adsorption) had been hospitalized two weeks previously for trauma, but there was nothing suggestive of infectious mononucleosis clinically or hematologically on either admission. The heterophile agglutination had been performed because of an atypical Wassermann reaction. Two patients had agglutinations of 1:320, one of which was adsorbed and fell to 1:160. The latter patient had had diphtheria three months previously, requiring tracheotomy, and was rehospitalized for what was considered to be postdiphtheritic neuritis. The other patient was hospitalized for a fever of unknown etiology, but no lymphadenopathy, splenomegaly, or characteristic blood pattern was noted during his short hospitalization. Eleven patients had titers of 1:160. The sera of ten of these were adsorbed and eight fell to 1:20 or lower, while two fell to 1:40. In all of these patients the diagnosis of infectious mononucleosis was excluded by a review of the history and the blood smears. There were also thirty-two patients in whom the titer was 1:80. The sera of ten of these were adsorbed, one remained 1:80, one fell to 1:40, two fell to 1:20, and six were negative. No adsorption test was carried out on the sera in the remaining nineteen cases. These data are also summarized in Table I.

The experimental material consisted of twenty-nine cases of Hodgkin's disease, ten of lymphosarcoma, two of reticulum cell sarcoma, two of reticuloendotheliosis, three of chronic granulocytic leucemia, six of chronic lymphocytic leucemia, one of acute leucemia, and two of giant follicle lymphoma. (See Table II.) One patient with gastric Hodgkin's disease had a titer of 1:160.

TABLE II. HETEROPHILE ANTIBODY AGGLUTINATION IN LEUCEMIC AND LYMPHOMATOUS DISEASES

	TOTAL	NEG.	1:10	1:20	1:40	1:80	1:160	OVER 1:160
Analysis of the Highest Titer for Each Individual								
Hodgkin's disease	29	12	2	8	4	2	1	0
Misc. leucemias and lymphomas*	26	14	3	5	3	1	0	0
Total lymphomas and leucemias, including Hodgkin's disease	55	26	5	13	7	3	1	0
Infectious mononucleosis	43	0	0	1	0	2	5	35
Analysis of All Tests Performed								
Hodgkin's disease	44	22	2	12	4	3	1	0
Misc. leucemias and lymphomas	36	19	5	8	3	1	0	0
Total lymphomas and leucemias, including Hodgkin's disease	80	41	7	20	7	4	1	0
Infectious mononucleosis	104	5	1	2	3	12	15	66

*Giant follicle lymphoblastoma (2), reticulum cell sarcoma (2), reticuloendotheliosis (2), lymphosarcoma (10), acute leucemia (1), chronic granulocytic leucemia (3), and chronic lymphocytic leucemia (6).

Unfortunately, no adsorption test was performed, but a re-examination one month later revealed a titer of only 1:20. Two patients with Hodgkin's disease had titers of 1:80, but no agglutination after adsorption with guinea pig kidney antigen. One patient with chronic lymphatic leucemia showed agglutination of 1:80 and a titer of 1:20 after adsorption. Therefore, only four patients or 7.3 per cent of the fifty-five patients with lymphomatous diseases had titers of 1:80 or higher.

DISCUSSION

There has been much study of the specific sheep cell agglutinin in the serum of patients with infectious mononucleosis since the original observation by Paul and Bunnell[3] in 1932. Although originally this agglutinin was thought to be the heterophile antibody of Forssman, differential adsorption studies have shown it to be a separate antibody, and similar studies have separated it from the antibodies of serum sickness. The selectivity of the test was first established by Bunnell[4] who found that in 1,600 Wassermann sera, with no diagnoses or ages available, only 0.8 per cent had titers of 1:128, and only 4 per cent had titers of 1:64.* Bernstein[5] found that in 300 hospital patients 14 per cent had final titers of 1:80, but that none were higher. Stuart[6] and co-workers found 6 per cent of normal controls positive at 1:80 final dilution, 0.66 per cent positive at 1:160, and 0.33 per cent positive at 1:320. In 412 miscellaneous cases, Butt and Foord[7] found that 5 per cent had titers of 1:32 and only 0.2 per cent had titers of 1:64 or more. Our results were similar; 7 per cent had titers of 1:80, 2.4 per cent had titers of 1:160, and 0.6 per cent had titers of 1:320 or higher. See Table III.

*In the original Paul-Bunnell technique, 0.5 c.c. of physiologic salt solution and 1.0 c.c. of a 1 per cent suspension of washed sheep red cells are added to 0.5 c.c. of diluted serum. Those authors reported their results in terms of the serum concentration before the salt solution and sheep cell suspension had been added. Current techniques are reported in terms of the final serum dilutions. Therefore, to compare the results of the two techniques it is necessary to multiply the titer of the Paul-Bunnell technique by a factor of four. In order to maintain uniformity, only the final serum dilution will be recorded in this paper.

The sensitivity of the test has been established by the demonstration that in the vast majority of cases of infectious mononucleosis, diagnosed by clinical and hematologic critera, the heterophile agglutination is positive in dilutions which do not overlap those of the normal control. It must be realized, however, that the demonstration of the selectivity and sensitivity of the test has made many clinicians reluctant to make the diagnosis without the confirmation of a positive heterophile agglutination. Thus, perhaps, the sensitivity of the test has been overrated because of this selection of cases. Davidsohn[8] reports 90 per cent of his thirty cases with positive reactions in final dilutions of 1:224 or

TABLE III. DISTRIBUTION OF HETEROPHILE ANTIBODY TITERS IN MISCELLANEOUS CASES

AUTHORS	NUMBER OF SERA	DISTRIBUTION					
Bunnell	1,600	1:64	4.0%	1:128	0.8%	1:256	0.0%
Bernstein	300	1:80	14.0%	1:160	0.0%		
Davidsohn		1:56	1.0%	1:112	0.0%		
Stuart and co-workers	300	1:80	6.0%	1:160	0.66%	1:320	0.33%
Butt and Foord	412	1:32	5.0%	1:64	0.2%	1:128	0.0%
Present series	458	1:80	7.0%	1:160	2.4%	1:320	0.6%

higher and 30 per cent positive in final dilutions of over 1:896. Bernstein found that in his sixty-five cases of infectious mononucleosis, 3 per cent had titers below 1:80, 5 per cent had titers of 1:80, and 92 per cent had titers of 1:160 or higher. On the other hand, Kaufman[9] found only 75 per cent of seventy-nine cases to have diagnostic titers, although he included titers as low as 1:28 if the differential adsorption did not alter the agglutination. Only four of Kaufman's seventy-nine patients had titers above 1:448. Kaufman's results are unusual in that the heterophile agglutination titers are quite low in patients diagnosed as having infectious mononucleosis. As previously noted, each case in our series which was accepted as infectious mononucleosis was verified by the presence of adequate clinical or hematologic data. In no case was the serologic reaction considered to be more than confirmatory. As noted in Table IV, 2.3 per cent had titers below 1:80, 4.6 per cent had titers of 1:80, 11.4 per cent had titers of 1:160, 9.1 per cent had titers of 1:320, and 72 per cent had titers of 1:640 or above. Thus, 72 per cent of the patients with infectious mononucleosis had titers higher than all other cases, with one exception, in which a heterophile test had been performed, and 93 per cent had titers in the diagnostic range. These data appear to compare favorably with those already cited, and they establish the sensitivity and selectivity of the method in use at this laboratory.

PREVIOUS OBSERVATIONS IN LYMPHOMATOUS DISEASES

No large series of observations has been reported on the nature of the heterophile antibody in the lymphomatous diseases. In their original report, Paul and Bunnell noted that in five patients with leucemic and lymphomatous diseases, the highest titer was 1:32 in a patient with myelogenous leucemia. In his supplemental report, Bunnell noted that in twenty-seven cases of Hodgkin's disease, agranulocytosis, miscellaneous leucemias, malignant thymoma, and benign lymphadenosis the highest titer was 1:32. Van Ravenswaay[10] observed fifteen patients, of whom ten had no heterophile antibodies, three had titers of 1:32, one had a titer of 1:64, and one had a titer of 1:128. Butt and Foord reported five cases of

TABLE IV. DISTRIBUTION OF HETEROPHILE ANTIBODY TITERS IN INFECTIOUS MONONUCLEOSIS*

AUTHOR	NUMBER OF CASES	TITER											
		0-1:8	1:14-1:20	1:28-1:32	1:56-1:64	1:80	1:112-1:128	1:160	1:224-1:256	1:320	1:448-1:512	1:640	1:896 AND OVER
Bunnell	15								27.7%		40.0%		33.3%
Davidsohn	30				3.5%		6.5%		6.5%		27.0%		56.5%
Bernstein	65		3.0%			5.0%		9.0%		12.0%		11.0%	60.0%
Butt and Foord	26	3.9%		3.9%	3.9%		15.5%		26.9%		11.5%		34.5%
Kaufman	79	11.4%	2.5%	20.3%	22.8%		24.1%		7.6%		6.3%		5.0%
Schultz	117 tests on 31 patients		0.8%	2.5%	5.0%		13.3%		35.8%		30.8%		9.2%
Present series	43		2.3%			4.6%		11.4%		9.1%		11.4%	60.5%

*Only the most strongly positive titer is represented, since many tests were performed after recovery when a significant titer would not be expected. In the cases cited by Schultz, such a breakdown was not possible.

lymphatic leucemia, four of myelogenous leucemia, two of aplastic and pernicious anemia, one of Hodgkin's disease, and one of myeloma, all of which were negative. Bernstein found one titer of 1:20 and another of 1:40, while the rest were negative in a group of seven miscellaneous lymphomas and leucemias. Kaufman found two titers of 1:112 and one of 1:224 in eleven lymphomas. All three had less than diagnostic titers after adsorption. Schultz found significant titers (1:56 or higher) in sixteen of eighteen tests on the sera of six patients with Hodgkin's disease, in none of eight tests on the sera of two patients with lymphatic leucemia, in six of eight tests on the sera of two patients with monocytic leucemia, in twenty-four of forty-five tests on the sera of six patients with myelogenous leucemia, and in nine of twenty-three tests on the sera of eight patients with sarcoma other than Hodgkin's disease. It is to be noted that this author did not establish the upper limits of normal for the test as performed in her laboratory. Since an analysis of the range of titers in patients having known infectious mononucleosis shows lower peak values than for the other series reviewed, except that of Kaufman (see Table IV), the normal and the diagnostic ranges may have an appreciable overlap. However, analysis of these titers of patients with proved infectious mononucleosis would suggest that the diagnostic titer should be 1:128 rather than 1:56. Such an interpretation would reduce the number of significant reactions in the lymphomatous diseases.

To determine the presence of a heterophile antibody in our cases of leucemia and lymphoma, we examined the sera of a group of fifty-five patients. As noted previously, only five of the eighty tests performed on the sera of these fifty-five patients fell within the "doubtful" or "diagnostic" range, as established for the technique and none was higher than 1:160. Four patients were represented by these five tests. Thus, only 7.3 per cent of the lymphoma group had tests which fell within the indeterminate range as compared with 10.0 per cent of a miscellaneous group not known to have either infectious mononucleosis or lymphoma. The data do not support the concept that there is an abnormally high titer for the heterophile antibody in patients with lymphomatous diseases. (See Table V.)

TABLE V. RECORDED HETEROPHILE ANTIBODY TITERS IN LYMPHOMATOUS DISEASES

AUTHOR	NUMBER OF CASES	COMMENT
Paul and Bunnell	5	One patient 1:32, others less
Bunnell	27	Highest titer 1:32
Van Ravenswaay	15	Three 1:32, one 1:64, and one 1:128
Butt and Foord	14	All negative
Kaufman	11	Two 1:112, one 1:224; all below diagnostic level after adsorption
Schultz	24 (102 tests)	Twenty-nine 1:56, nineteen 1:112, five 1:224, two 1:448
Present series	55	Seven 1:40, three 1:80, one 1:160

CONCLUSIONS

1. Eight hundred and seventeen heterophile agglutination tests were performed on the sera of 556 patients. These patients include forty-three cases of infectious mononucleosis, fifty-five cases of miscellaneous lymphomatous diseases, and 458 unspecified cases for routine tests.

2. Of 458 patients with miscellaneous disorders, only 10.0 per cent had titers of 1:80 or higher and 3.0 per cent had titers of 1:160 or higher. Three patients, 0.6 per cent, had positive titers of 1:320 or higher.

3. One hundred and four tests were performed on the sera of the forty-three patients with infectious mononucleosis. Ninety-three had at least one titer of 1:160 or higher, and 98 per cent had at least one titer of 1:80 or higher. Thirty-five patients, 81 per cent, had titers of 1:320 or higher.

4. After adsorption of the most strongly positive serum with guinea pig kidney antigen, all but one of twenty-eight patients with infectious mononucleosis had titers of 1:160 or higher. With but three exceptions, the sera of twenty-five patients with titers of 1:80 or higher, but in whom infectious mononucleosis could not be established, showed titers of 1:40 or lower after adsorption.

5. Twenty-nine patients with Hodgkin's disease and twenty-six with miscellaneous lymphomatous and leucemic diseases were tested. Four patients, 7.3 per cent, had five tests in the borderline range of 1:80 and 1:160. Three of these patients had tests that fell into the negative group after adsorption. One patient did not have a differential adsorption test performed, but a subsequent test was well below diagnostic level.

6. There is no evidence in this series to indicate that there is a higher titer for the heterophile antibody in patients with lymphomatous diseases than there is in an unselected group of patients with no evidence of infectious mononucleosis.

7. The data indicate that the heterophile agglutination test, as routinely performed in this laboratory, is sensitive and selective. The reliability of the test is increased by considering both the 1:80 and 1:160 dilutions in the doubtful range, and by supplementary adsorption with guinea pig kidney antigen.

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LIVER FUNCTION TESTS IN INFECTIOUS MONONUCLEOSIS

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STUDIES of hepatic structure[1-10] and function[11-19] have provided evidence that hepatitis occurs in the majority of patients with infectious mononucleosis. Several series of patients, most with elevated heterophile agglutinin titers, have been investigated utilizing various combinations of liver function tests. In these early reports, attention was paid to particular tests, or observations on a majority of patients were made at only one time during the illness using a battery of tests. The present study was designed to provide additional data on the course as well as the incidence of hepatic dysfunction in infectious mononucleosis by utilizing a number of tests repeated at intervals during the disease.[16]

MATERIALS AND METHODS

Serial tests* of liver function were made in twenty-four consecutive cases of infectious mononucleosis. The subjects consisted of twenty-three hospitalized patients and one student followed at home. There were fourteen women and ten men. Ages ranged from 17 to 36 years with an average of 22 years. Two of the patients were jaundiced. The diagnosis was based on the usual clinical criteria plus the demonstration of agglutinins for sheep erythrocytes to a titer of 128 or above as determined by the method of Stuart.[20]

Total serum bilirubin was determined by the technique of Malloy and Evelyn[21] and 1.0 mg. per cent was taken as the upper limit of normal. Urine urobilinogen was measured in a two-hour afternoon specimen by the method of Watson and co-workers[22]; more than 1.0 total Ehrlich unit was considered abnormal. Cephalin-cholesterol flocculation tests were done by the technique of Hanger[23] and a reaction of 2+ or more in forty-eight hours was considered to be abnormal. Thymol turbidity tests were done by a modification of the Maclagan[24] method in which normal sera gave reactions up to only 2 units. Serum alkaline phosphatase was determined by the technique of Shinowara, Jones, and Reinhart[25]; values of 12 units and over were considered to be elevated. In measuring bromsulfalein excretion, 5 mg. per kilogram of body weight were injected and blood was drawn in thirty and forty-five minutes. Five per cent retention of the dye in forty-five minutes was considered abnormal.

RESULTS

Some degree of hepatic dysfunction, as demonstrated by abnormal values for one or more tests, was present in the two jaundiced patients and in twenty-one of the twenty-two nonjaundiced patients. The results obtained in the twenty-four cases are summarized in Table I.

Thymol turbidity values were elevated in twenty, 83 per cent, of the twenty-four cases. The values ranged from 2.1 to 4 units in fifteen cases and above 4

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*These tests were performed in the clinical laboratories of University Hospitals under the direction of Dr. J. W. Price.

TABLE I. RESULTS OF LIVER FUNCTION TESTS IN CASES OF INFECTIOUS MONONUCLEOSIS

TEST	NUMBER OF PATIENTS TESTED	NUMBER OF PATIENTS WITH ABNORMAL VALUES	PER CENT OF PATIENTS WITH ABNORMAL VALUES
Thymol turbidity	24	20	83
Cephalin flocculation	24	19	79
Bromsulfalein	17	12	71
Urine urobilinogen	16	9	56
Alkaline phosphatase	21	8	38
Serum bilirubin	22	4	18

units in five cases. The patients with increased thymol turbidity values had abnormal cephalin-cholesterol flocculation reactions except for one patient in whom the test was unsatisfactory.

The results of the cephalin-cholesterol flocculation test were abnormal in nineteen of the twenty-four cases, corresponding closely with the thymol turbidity test. Five patients had peak values of only 2+; the remaining patients developed 3+ to 4+ reactions. In five patients with normal flocculation reactions, bromsulfalein retention and increased alkaline phosphatase occurred in two, abnormal values for bromsulfalein and thymol turbidity occurred in one, bromsulfalein retention alone was present in one, and there were no abnormal findings in one.

Decreased bromsulfalein excretion was found in twelve of seventeen patients tested, the values ranging from 5 to 45 per cent retention of the dye in the forty-five minute specimens. In one instance, 10 per cent dye retention was the only abnormal finding.

Serum alkaline phosphatase was elevated in eight of twenty-one patients, or 38 per cent. In four other cases values ranged from 8 to 12 units and showed subsequent falls. An elevation of alkaline phosphatase, in one instance to as high as 56 units, occurred in two patients with normal cephalin flocculation and thymol turbidity values. Elevation of alkaline phosphatase, however, usually was associated with changes in other tests indicative of hepatocellular disease. Bromsulfalein retention was associated with an increase of alkaline phosphatase in six of seven cases. Four of seven patients had elevated quantitative urine urobilinogen values. Findings commonly associated with obstructive jaundice were uncommon in the patients with elevated alkaline phosphatase. No increased cholesterol values were found; only one of these patients had an increased serum bilirubin.

The total serum bilirubin was elevated in four of twenty-two patients, two of which were clinically jaundiced. Quantitative urine urobilinogen values were increased in nine of sixteen cases. These abnormalities were always associated with other changes.

No abnormal values for total proteins and albumin-globulin ratios were found in eighteen cases. Serum cholesterol values were normal in the eleven patients tested. Borderline reduction in prothrombin time was found in two of sixteen cases.

In the twenty-three patients with abnormal values, the results of only one test were abnormal in three, the results of two tests were abnormal in four,

and the remaining sixteen patients had changes in three or more tests indicative of impaired hepatic function.

Since few patients were tested during the first week of disease, and others were not admitted until the third or fourth weeks of disease, the trend of the

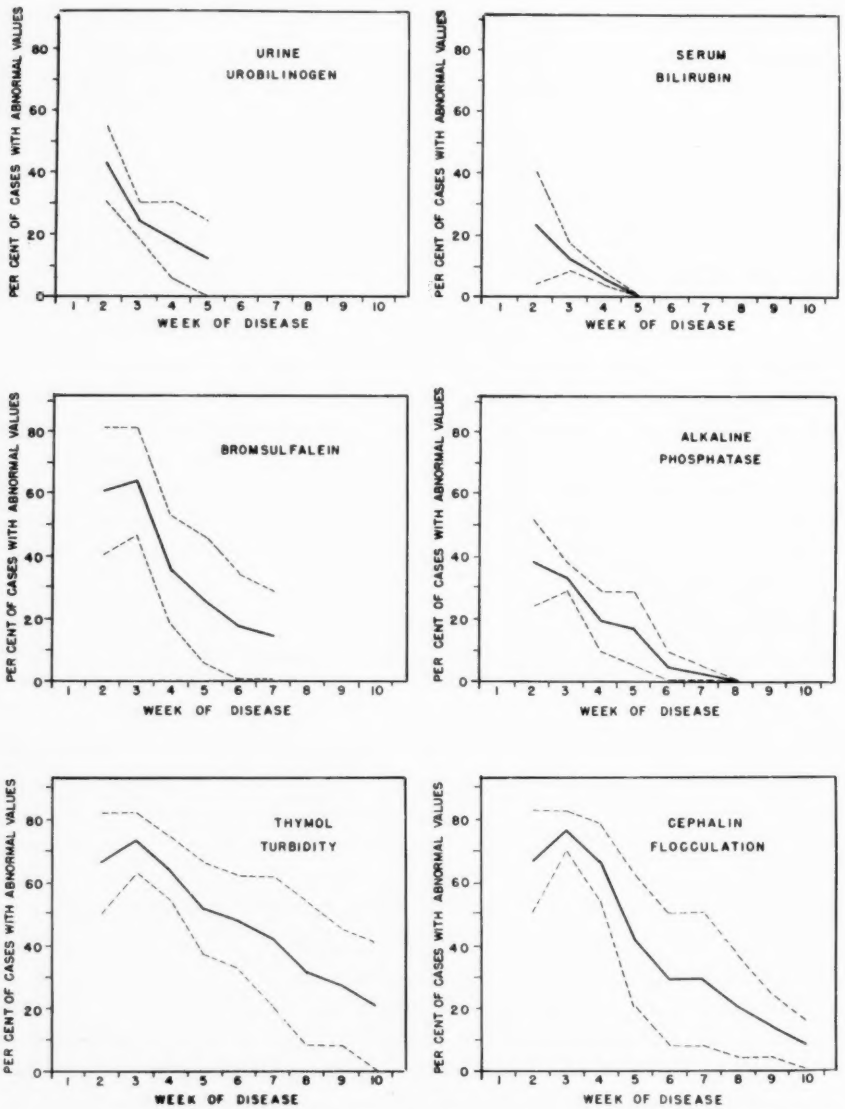

Fig. 1.—Method of estimating the results of liver function tests in all cases by week of disease (see text for explanation).

changes measured by each test could not be determined from onset of first symptoms. The duration of abnormality could not be measured absolutely because, following discharge, intervals of two and sometimes four weeks separated serial determinations. An approximation of the course and duration of the abnormality reflected by each of the six tests, however, was obtained as follows.

The number of patients with an abnormal value during a given week was plotted (as per cent of patients tested). Patients with normal values were subtracted from the remainder to obtain the number of cases in which the results of the test during that week were unknown. The number of patients with unknown values was then added to the number of patients with abnormal values, giving the maximum number of patients that could have had abnormal values. These cases (as percentages) were plotted above the ones with known abnormal values. The bands obtained for each test (Fig. 1) indicated the possible range of abnormal values, and as more patients returned, the number unknown decreased. For example, by the tenth week all but four patients were known to have normal cephalin flocculation values; all patients tested were followed until they had normal alkaline phosphatase values.

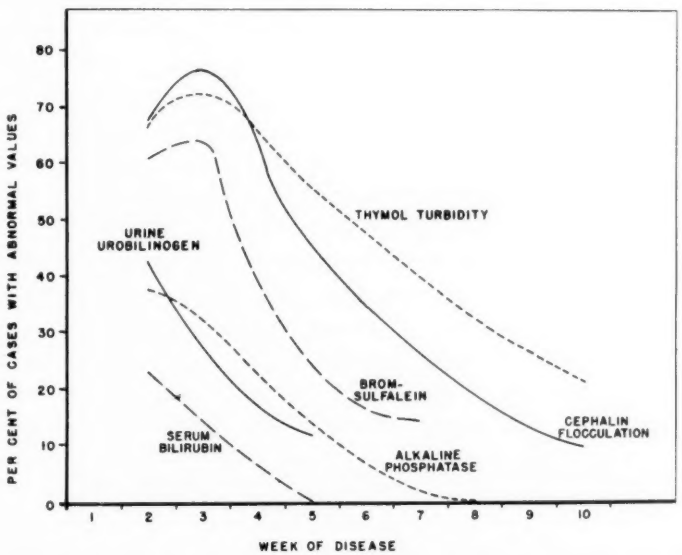

Fig. 2.—Approximate per cent of cases with abnormal values for liver function tests by week of disease (see text for derivation of these curves).

With the limits thus defined, curves were drawn through the mid-points of the bands to give an approximation of the frequency of occurrence and duration of these abnormal values by week of disease (Fig. 1). These curves were then smoothed by inspection as shown in Fig. 2. The peaks shown correspond closely with the over-all results shown in Table I, and it is felt that the curves represent the rate of improvement of hepatic function as measured by the various tests.

Values for serum bilirubin, urine urobilinogen, and alkaline phosphatase were maximum during the second week and returned to normal by the fifth to eighth week of disease. The incidence of increased bromsulfalein retention and abnormal cephalin flocculation and thymol turbidity reactions was greatest during the third week; the number of cases showing abnormal values then decreased at approximately the same rate. About one-sixth of the cases still showed bromsulfalein retention during the sixth to seventh week of disease.

Cephalin flocculation and thymol turbidity values were the last to return to normal, being elevated in 20 and 30 per cent of cases respectively at the end of eight weeks. Elevated thymol turbidity values were the most persistent finding. Indeed, the persistence of altered thymol turbidity reactions coupled with the decreasing enthusiasm of the patient for repeated visits accounts for most of the patients who were not followed until the values for each test had returned to normal. The slope of the curves suggests that all patients would have had normal values within three months.

The approximations detailed permit the following generalizations. During the second and third weeks of disease, two-thirds to three-fourths of patients with infectious mononucleosis have laboratory evidence of hepatic disease. At five weeks, multiple abnormalities are still detectable in one-third to one-half of the patients. After two months, only changes in cephalin flocculation and thymol turbidity reactions are still present in 20 to 30 per cent of the patients, and these changes disappear by the third month.

An attempt was made to correlate the occurrence of changes in liver function tests with signs and symptoms of hepatic involvement. Hepatomegaly was present in eleven patients, four of which had tenderness in the right upper quadrant. One patient had tenderness without hepatomegaly. These findings were not correlated with the degree of abnormality or the total number of abnormalities. Symptoms possibly referable to hepatitis occurred as follows: anorexia in twenty cases, nausea in nine, and vomiting in eight. Such symptoms, particularly anorexia, may, of course, be nonspecific and due to pharyngeal involvement or systemic constitutional effect. The number or intensity of symptoms showed no correlation with the dergee of hepatic dysfunction indicated by laboratory tests. One of the patients with clinical icterus and two other patients having abnormal values in five different tests complained only of anorexia. One patient had all of the symptoms listed, yet showed minimal laboratory changes.

Similarly, the height of the heterophile antibody titer was not related to the changes in the liver function tests.

DISCUSSION

Few of the laboratory tests described in this and other reports actually measure the normal metabolic activities of the liver and are not, strictly speaking, function tests. However, abnormal values for these tests are generally accepted as indicative of hepatocellular disease and impaired liver function. It is apparent that some degree of hepatic dysfunction, as evidenced by these tests, occurs in the majority of cases of infectious mononucleosis. In the original study of nonjaundiced cases of infectious mononucleosis, Cohn and Lidman[11] demonstrated impairment of liver function by two or more tests in all of fifteen patients.

That the pathologic changes in infectious mononucleosis are not restricted to the throat, lymph nodes, and spleen has become increasingly apparent. Involvement of many different organs in individual cases may be manifest by skin

rashes, purpura, and epistaxis,[26-29] neuritis, meningitis, and encephalitis,[9, 30-32] myocarditis,[33] and pneumonitis.[34, 35] It is not surprising, therefore, that the liver is involved in such a generalized disease.

Jaundice was noted in single cases of infectious mononucleosis in this country by Downey and McKinlay[36] in 1923 and Mackey and Wakefield[37] in 1926. The latter authors advanced the theory that the most probable cause of the jaundice was lymph node enlargement obstructing the common bile duct. Two years later, Mason[38] described two additional cases and suggested that an acute hepatitis was a more reasonable explanation for the high serum bilirubin. But the concept of lymph node obstruction persisted until as recently as 1944.[39] In that year three reports[40-42] estimated that from twenty-one to thirty-five cases of jaundice in infectious mononucleosis had been reported. Since that time the total has risen to approximately one hundred cases, and the incidence of jaundice in different series has ranged from 0 to 13 per cent.[43]

In 1942, Kilham and Steigman[1] obtained a punch biopsy from a jaundiced patient and described a focal acute hepatitis. Van Beek and Haex[2] observed similar histologic changes in a biopsy specimen from a patient with mononucleosis but no jaundice. Studies of seven additional biopsy specimens and thirteen autopsied patients have been reported,[3-10] the livers of patients both with and without jaundice showing similar morphologic changes.

To emphasize the occurrence of alterations in liver function tests in nonjaundiced patients with infectious mononucleosis, the results of the previous studies in such patients and those of the present report are summarized in Table II. It is evident that such a battery of liver function tests will reveal abnormalities in a high proportion of hospitalized patients with infectious mononucleosis. This may not necessarily mean that all such patients have hepatitis. Abnormalities of these tests occur in such diverse conditions as rheumatoid arthritis and malaria,[44, 45] pneumonia,[46] measles,[18] heart failure,[47] hyperthyroidism,[48] and hyperthermia.[49] Hicks, Holt, Guerrant, and Leavell[50] have reported a correlation between the percentage of bromsulfalein retention and the height of the temperature during artificially induced fever. The same authors also described abnormal cephalin-cholesterol flocculation reactions in the sera of patients convalescent from primary atypical pneumonia. It has been suggested[51] that the flocculation phenomena observed in infectious mononucleosis are associated primarily with alterations in serum proteins and lipids and are related to liver changes only insofar as the latter contribute to alterations in these serum components. The same authors also noted elevation of the alkaline phosphatase, occasionally pronounced, in nonjaundiced patients. Because most patients with infectious mononucleosis were not jaundiced, it was postulated that the mechanism responsible for the hyperphosphatasemia may not be identical with that in infectious hepatitis.

The bulk of the evidence indicates, however, that the alterations in these tests reflect an inflammatory process in the liver. Control studies in patients with upper respiratory infections have shown only infrequent alterations in these tests.[14, 15] In the present series, changes in bromsulfalein excretion or

TABLE II. LIVER FUNCTION TESTS IN 301 CASES OF INFECTIOUS MONONUCLEOSIS WITHOUT JAUNDICE AS REPORTED BY VARIOUS AUTHORS

AUTHORS	NUMBER OF CASES	ICTERIC INDEX OR BILIRUBIN			URINE UROBILINOGEN			CEPHALIN-CHOLESTEROL FLOCCULATION			THYMOL TURBIDITY			ALKALINE PHOSPHATASE			BSP RETENTION			COMMENT
		NO.*	+†	%	NO.	+	%	NO.	+	%	NO.	+	%	NO.	+	%	NO.	+	%	
Cohn and Lidman[11]	15	15	0	0	0			0			15	15	100	8	7	88	15	15	100	Impairment indicated in all cases by 2 or more tests
Carter and Maclagan[45]	18	0			0			0			18	10	56	0			0			Also found abnormal values in malaria, rheumatoid arthritis, heart failure and endocarditis
Gall[12]	33	19	8	42	0			25	23	92	14	13	93	33	28	85	0			Some degree of interference with function in 31 of 33 patients
DeMarsh and Alt[13]	19	19	5	26	0			18	15	83	0			0			19	15	79	Some evidence of functional impairment in all cases
Evans[14]	19	19	0	0	0			19	17	89	19	13	68	14	6	43	1	1	100	Evidence of abnormality in 18 of 19 cases; negative results in controls
Peterson[15]	39	39	15	38	39	21	54	39	26‡	67	39	18	46	11	8	73	32	19	59	Evidence of impairment in 20 of 39 cases by 3 or more tests
Brown, Sims, White, and Clifford[16]	83§	74	28	38	68	23	34	82	70	85	77	38	49	0			41	20	49	Results of 1 or more tests abnormal in 75 of 83 cases
Ernst and Dotti[17]	9	0			0			0			9	8	89	0			0			Also found abnormal values in virus pneumonia, secondary syphilis, malaria, sickle cell anemia
Iversen and Raaschou[18]	24	33	0	0	33	0	0	0			33	24	73	0			0			Thymol turbidity also increased in 71% of cases of measles
Angle[19]	20	11	4	36	0			19	13	68	0			0			17	13	76	Evidence of functional impairment in 16 of 20 cases
Jordan and Albright	22	20	2	10	14	7	50	22	17	77	22	18	82	20	7	35	17	12	71	Some abnormality in 21 of 22 cases; impairment by 3 or more tests in 16 of 22 cases
Total	301	249	62	25	154	51	33	224	181	81	246	157	64	86	56	65	142	95	67	

*Number of cases in which test was done.
†Number of cases with abnormal values.
‡From author's tabulated data.
§No statement regarding clinical jaundice.

other abnormalities were not related to the presence of fever. Electrophoretic studies of serum from patients with mononucleosis have shown abnormalities similar to those observed in infectious hepatitis and cirrhosis.[52] Furthermore, evidence of hepatitis has been obtained in both jaundiced and nonjaundiced patients.[1-10] It would be unwise, however, to interpret any isolated finding as indicative of hepatitis; rather, composite serial studies should be performed before concluding that liver involvement is present.[53] The present and other composite studies have revealed multiple abnormalities of liver function in approximately two-thirds to three-fourths of cases of infectious mononucleosis.

The occurrence of such abnormalities raises the question of their course and significance as related to the duration and prognosis of hepatitis in infectious mononucleosis. In the approximate 10 per cent of patients which have developed jaundice, the jaundice has been minimal and of short duration, the average duration ranging from one to three and one-half weeks in various series.[29, 35, 42, 54-56] Wechsler[29] reported three patients with jaundice for over forty days, the longest duration of icterus being forty-eight days. Abrams[43] observed a patient who was visibly icteric for eleven weeks.

In the nonicteric cases, one or more abnormal values have persisted for two to six weeks in the majority of reported cases. As might be expected from the similar nature of the pathologic picture, the appearance and trend of the changes in the various tests resemble those seen in infectious hepatitis.[53, 57] In the present study, the maximum number of changes was observed during the second and third weeks of disease, and multiple abnormalities were still present in one-third to one-half of the patients during the fifth week of disease. Changes in the cephalin flocculation and thymol turbidity reactions were still present in about one-fourth of the patients after two months. In three of DeMarsh and Alt's cases,[13] tests remained positive for two, three, and four and one-half months. Cohn and Lidman[11] stated that one of their patients had developed chronic hepatitis.

Thus hepatitis frequently occurs in infectious mononucleosis as a mild, nonicteric, usually transient process demonstrated by liver function tests. Jaundice, sometimes prolonged, does occur, and hepatic involvement may occasionally be more than a transient phenomenon. As in infectious hepatitis,[57-61] the possible relationship of hepatitis in infectious mononucleosis to the pathogenesis of cirrhosis must now be considered.

SUMMARY

Abnormalities in one or more serial liver function tests occurred in twenty-three of twenty-four cases of infectious mononucleosis. Sixteen cases showed changes in three or more tests.

Elevated cephalin-cholesterol flocculation and thymol turbidity values were found in 79 and 83 per cent of the patients respectively. Abnormal bromsulfalein excretion was demonstrated in 71 per cent, and the alkaline phosphatase was elevated in 38 per cent of those tested.

‡From author's unpublished data.
§No statement regarding clinical jaundice.

The maximum number of changes was observed during the second and third weeks of disease. Altered cephalin flocculation and thymol turbidity reactions were the most persistent abnormalities, lasting more than two months in a fourth of the patients.

The occurrence of abnormal liver function tests was not correlated with the signs and symptoms of hepatic involvement or with the height of the heterophile antibody titer.

Composite serial studies indicate that approximately two-thirds to three-fourths of patients with infectious mononucleosis have hepatitis.

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THE EFFECTS OF INTRAVENOUS ADMINISTRATION OF COMMERCIAL BEEF HEPARIN ON THE THROMBOCYTE COUNT AND HEPARIN SENSITIVITY IN MAN

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SMALL amounts of commercial heparin added in vitro to blood from patients with thrombocytopenic purpura result in a delay in coagulation greater than is encountered in normal blood with a similar amount of heparin. This has been demonstrated by heparin-protamine titration[1] and in the Waugh-Ruddick test.[2] The reason for this increased sensitivity to heparin has not been explained adequately. Copley and associates[3, 4, 5] injected heparin into dogs, mice, rabbits, and hamsters and noted a decrease in the number of platelets in the peripheral blood as well as the appearance of petechial hemorrhages. They also noted the formation of emboli which were apparently composed of platelet agglutinates to which a few white cells adhered. Copley[6] postulated that the agglutination of the platelets and the formation of emboli were responsible for both the thrombocytopenia and the appearance of petechial hemorrhages which followed the injection of heparin. Certain aspects of this work in animals were confirmed by Fidlar and Jaques[7] and also by Quick and associates.[8] These two groups of investigators have recently studied the effects of intravenous injections of heparin on the platelet count in human subjects. Quick found no reduction in the platelet count in two human subjects following the intravenous injection of heparin, although agglutination of platelets and thrombocytopenia invariably occurred in dogs. Fidlar and Jaques reported a drop in the platelet count in one human subject following the administration of heparin.

By means of the Waugh-Ruddick test[9, 10] we have shown that while they are actively bleeding the blood of patients with thrombocytopenic purpura is unusually sensitive to heparin.[2] In an attempt to explore further the relationship between the platelet count and the heparin sensitivity, we did platelet counts before and at various intervals after the intravenous administration of commercial heparin and, by means of the Waugh-Ruddick test, compared the action of small amounts of heparin added in vitro to blood previously heparinized in vivo. Thrombocytopenia was not present in any of the patients nor did they exhibit any hemorrhagic tendency.

METHODS

The Waugh-Ruddick test was performed prior to the administration of heparin and again when the effect of the heparin was at its height. The technique of the test is as follows.

Nine Wassermann tubes (100 by 13 mm.), thoroughly cleaned and dried, are placed in a rack. Tube number one remains empty until the blood is added.

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Tube number two contains ½ c.c. of 9/10 per cent normal saline. Serial dilutions of heparin in ½ c.c. of saline are added to the remaining seven tubes so that tube three receives 0.1 unit of heparin in ½ c.c. saline, tube four receives 0.2 unit of heparin in saline, and so on until tube nine receives 0.7 unit of heparin. To each of the tubes is added 1 c.c. of blood drawn with a minimum of trauma, using a number eighteen needle and measured from the same 10 c.c. syringe in which it was drawn. The tubes are stoppered and tipped to approximately 85 degrees at two-minute intervals until coagulation occurs. The time which elapses from the moment blood is first seen in the syringe until coagulation occurs is the end point in that particular tube.

Platelet counts were made prior to the administration of heparin, from four to eleven minutes after the administration, and again at the time the Waugh-Ruddick test was performed which was from thirty to forty minutes after the heparin injection. Platelet determinations were made as follows.

Venous blood was drawn from the antecubital vein using a saline-rinsed or oil-coated syringe by means of an 18 gauge needle. With the needle removed, the blood was carefully discharged in a small pool on a clean glass plate and immediately drawn to the 1.0 mark in two red blood cell pipettes which contained a small quantity of diluting fluid. The blood was then diluted to 1 part in 100 with a mixture prepared as follows: 40 per cent formaldehyde, 6.0 c.c.; sodium oxalate, 1.6 Gm.; crystal violet, 0.01 Gm.; distilled water added to make 100 cubic centimeters.

All platelet counts were made in duplicate and the results given in the table are the averages.

The clotting time of the whole blood was determined before the administration of heparin and again after a forty-minute interval, at the time the Waugh-Ruddick test was performed. On two patients it was determined ten minutes after the injection of heparin, at the time that a platelet count was performed. Clotting times were done by the Lee-White method.

Standard commercial beef heparin obtained from two pharmaceutical manufacturers was used. It was measured and injected by means of standard 1 or 5 c.c. syringes, depending on the amount to be administered. The manufacturer's statement as to its potency was utilized for all calculations. From 9 to 50 mg. were administered to each patient, as is shown in the table.

TABLE I

	HEPARIN (MG.)	INITIAL PLATELET COUNT (THOUSANDS)	PLATELETS IN THOUSANDS — MINUTES AFTER INJECTION			CLUMPS	WHOLE BLOOD CLOTTING TIME		
			4	10	40		BEFORE	10 MIN. AFTER INJECTION	40 MIN. AFTER INJECTION
1	50	225		208	205	None	10	40	68
2	10	201		204	234	None	6	24	14
3	10	174	178		192	None	8		20
4	10	236	254		240	None	6		12
5	8	246	267		246	None	10		14
6	18	193	160		143	Few	12		64
7	9	325		309		None	16		36
8	9	182		192		None	12		36
9	10	288		264		None	10		22
10	19	267		246	146	Few			
11	27	96		103	117	None			
12	50	153		163	138	None	6		26
13	50	355		397	405	None	5		27
14	50	169		153	150	None	5	55	41

RESULTS

As will be noted in Table I, there was no consistent change in the platelet count following the intravenous administration of heparin in dosages ranging from 9 to 50 milligrams. The variations in the counts before and after the administration of heparin are within the limits of error for the method and show no consistent increase or decrease from the control count taken before heparin was administered. An occasional small clump of platelets was noted in two

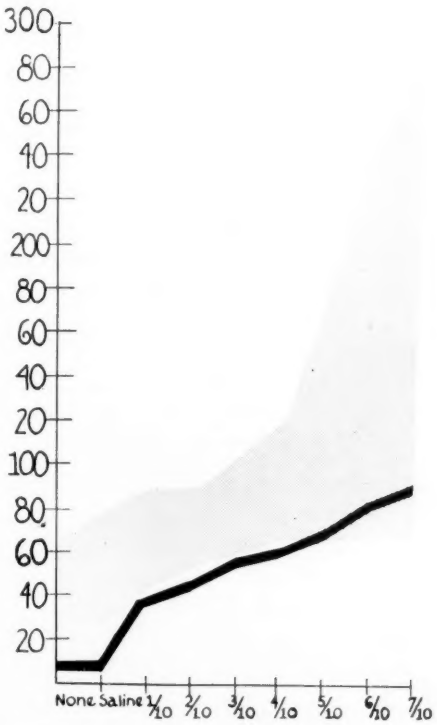

Fig. 1.—The heavy line shows the average Waugh-Ruddick curve prior to heparin administration. The shaded area represents the extremes in response obtained after intravenous administration of heparin.

patients after the heparin was given, but this phenomenon was not encountered in the others. The clumping of platelets after the administration of heparin was not confirmed by this method either by the finding of clumped platelets or by a reduction in the platelet count.

The clotting time of the blood was consistently prolonged following the administration of heparin. The degree of prolongation varied to some extent in individual patients receiving the same amount of heparin but tended to be more prolonged in those receiving larger amounts. There was no correlation between the prolonged coagulation time and the platelet count.

The Waugh-Ruddick test when performed on nine subjects prior to the injection of heparin was within the limits of normal. The time of coagulation

in tubes 1 and 2 which contained no heparin varied from 6 to 16 minutes. A gradual increase in the time required for coagulation was noted in the tubes to which heparin was added, the time lengthening as more heparin was present, but coagulation ultimately occurred in all cases in from 60 to 150 minutes. The Waugh-Ruddick test was repeated forty minutes after the administration of heparin in nine patients and in three instances it was also done ten minutes after heparin was given. As can be seen in the table, the coagulation time was prolonged after the forty-minute interval. The curve of the Waugh-Ruddick test, done at this time, followed in general the same course as in the control except that the initial readings were higher, i.e., the coagulation time was prolonged (Fig. 1). In the subsequent tubes coagulation was prolonged, in most instances, as compared with the control curve in the same patient, but this was not always true. In some cases, heparin in vivo had no apparent effect except in tubes 1 and 2. In no instance was the blood rendered incoagulable under the conditions of this test by the injection of heparin. We have shown previously that the blood drawn from patients with thrombocytopenic purpura who were actively bleeding becomes incoagulable when subjected to the effect of heparin in the Waugh-Ruddick test. Since this does not occur with the administration of commercial heparin, it is an additional point against the hypothesis that thrombocytopenic purpura is a heparinoid state.

SUMMARY

The intravenous injection of commercial beef heparin into normal human subjects did not produce thrombocytopenia nor did it cause an appreciable degree of clumping of the platelets.

The curve obtained by the Waugh-Ruddick test did not resemble the curve obtained in patients with thrombocytopenic purpura, such as were previously reported, nor did commercial beef heparin render the blood incoagulable under the conditions of this test.

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CONTROL OF HEART RATE WITH AN INTRACARDIAC THERMODE

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CLASSICAL experiments of McWilliam,[1] Flack,[2] and others have shown that the rhythmicity of the sino-atrial node of various animals can be altered by thermal changes in excised and perfused hearts, and in the exposed heart in situ. We have devised a special thermode which can be placed in the region of the S-A node by venous (jugular, brachial) catheterization[3] of the intact experimental animal or man.

METHODS

The thermode consists essentially of a 4 by 30 mm. copper U tube attached to the cardiac end of a double lumen catheter. In a later model designed for use in man the thermode was chrome plated and measured 3 by 30 millimeters. The thermode temperature is regulated by perfusing water of various temperatures (4 to 60° C.) through this closed system. In five intact dogs, anesthetized with pentobarbital, changes in heart rate and in the form of the electrocardiogram were determined by continuous tracings recorded before, during, and after thermal changes. Standard and augmented "unipolar" limb leads and intracavitary leads were employed. The exact position of the thermode was confirmed by careful necropsy examination. In over 100 experiments it was possible to vary the rate at will, as was done in the experiments on exposed hearts already mentioned. No attempt was made to maintain a change in rate for periods exceeding fifteen minutes.

RESULTS

The most pronounced effects occurred when the thermode was located at the right border of the junction of the superior vena cava and right atrium (Fig. 1). Detailed anatomic studies[4] have shown that the S-A node is situated in this region. In our experiments, the catheter thermode was considered to be located in the approximate region of the S-A node. "Unipolar" electrocardiograms recorded from the tip of the thermode showed characteristic negative P waves exceeding QRS in amplitude. Clinically similar complexes are obtained when the tip of a cardiac catheter electrode is moved under fluoroscopic control to the level of the upper right atrium and lower superior vena cava. There was a latent period of two to twenty-five seconds (Fig. 2) which probably represents the lag inherent in our system for changing the temperature of the thermode and adjacent areas and does not indicate refractoriness of the S-A node. Greater extremes of temperature of perfusion fluid were required because of the difficulty of locating precisely the region

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of the S-A node, and because of the damping effect of the circulating blood surrounding the thermode. In more exact studies in exposed hearts beating in situ, Ganter and Zahn[5] demonstrated the thermal sensitivity of the S-A node and checked their results histologically. Later it was found that the S-A node responds to changes in temperature of 1 to 2° C.[6]

Perfusion of our intracardiac thermode with relatively cold water (4 to 25° C.) caused the sinus rate to decrease from the control range of 120-150 to 80-90 beats per minute (Fig. 3). When the region of the S-A node was cooled excessively, the pacemaker shifted to the A-V node, with a rate varying from 35 to 86 per minute (Fig. 4). The heart rate at the time this transfer occurred varied in individual dogs.

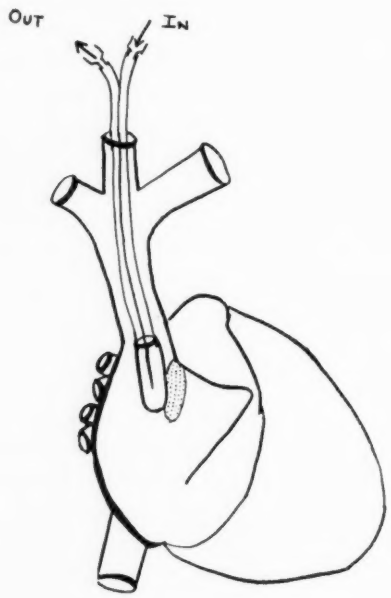

Fig. 1.—Diagram of right lateral view of dog's heart. Double lumen cardiac catheter thermode is near S-A node (stippled area).

Warm perfusion (45 to 55° C.) increased the sinus rate to a maximum of 200 to 232 per minute (Figs. 2, 3, and 4). The acceleration produced by heating was relatively greater and persisted longer than the slowing produced by cooling.

There were other electrocardiographic changes. The Q-T interval (electrical systole) was shortened by hot and lengthened by cold perfusion of the thermode. The P-R interval was usually unaltered but increased 0.01 second in several experiments when excessive sinus rates (230) were produced. No significant change in the duration or amplitude of the QRS complex was observed. In most experiments the T wave was unaltered despite marked

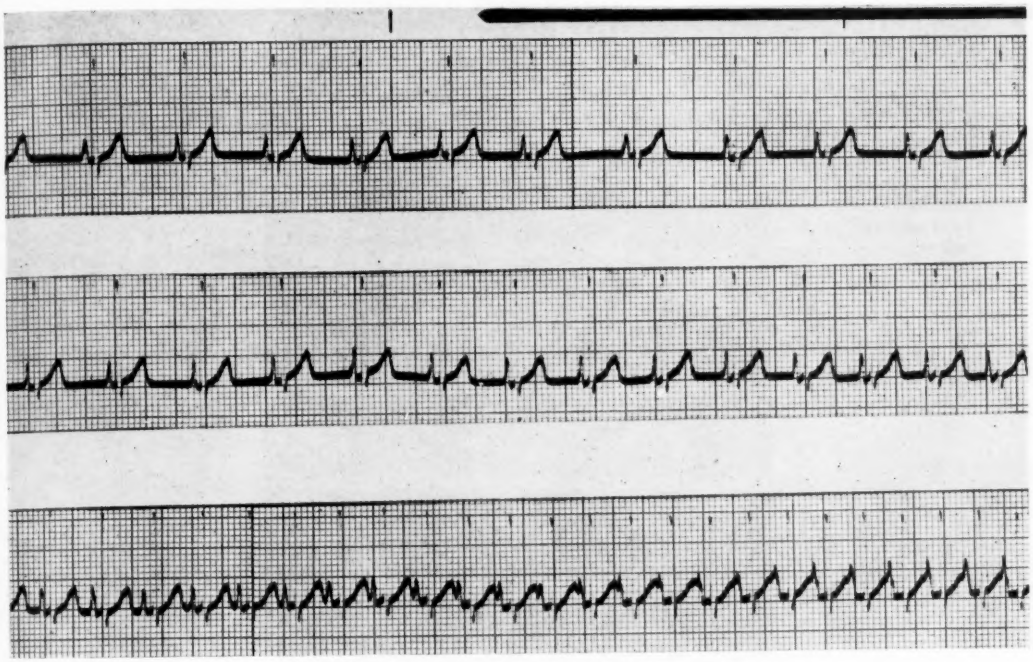

Fig. 2.—Lead II, continuous record. Lag period between onset of perfusion of thermode with 55° C. water (black marker) and attainment of maximal acceleration, third row.

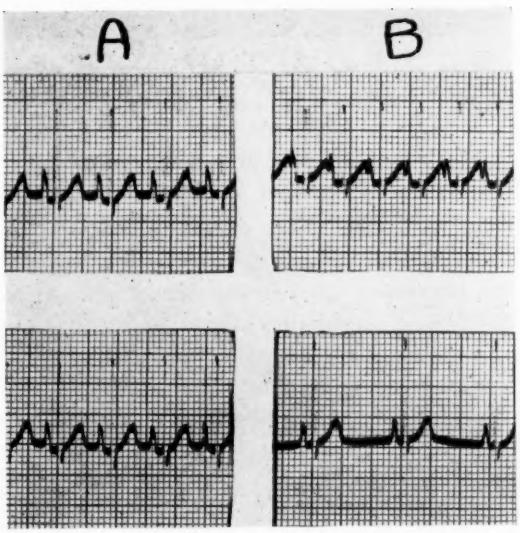

Fig. 3.—Lead II. Effect of warm (55° C.) and cold (15° C.) perfusion of intracardiac thermode. Upper row: heat increased rate from control (*A*) 166 beats per minute to 231 (*B*). Lower row: cold slowed rate from control (*A*) 162 beats per minute to 97 (*B*).

changes in rate. When perfusion was rapid, however, there were occasional T-wave changes. This indicated that there had been sufficient changes in the temperature of the blood flowing by the thermode to alter the

rate of repolarization of the subendocardial lamina of the ventricles.[7]* Thus, in accordance with our previous observations, cold perfusion of the thermode resulted in large negative T waves in cavity leads and in tall positive T waves in extracavitary leads; heating was accompanied by oppositely directed T waves.

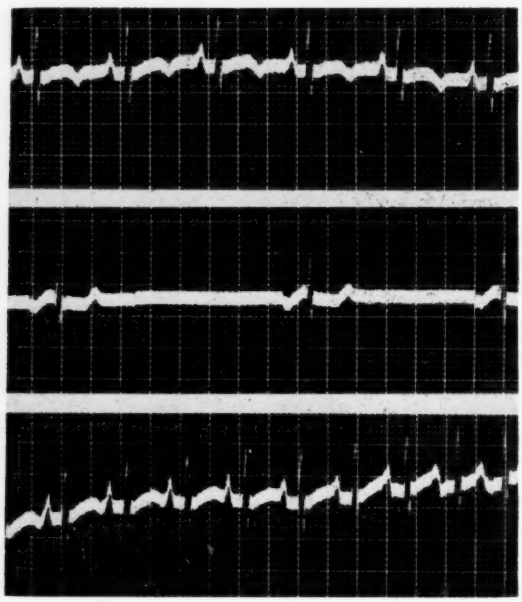

Fig. 4.—Effect of excessive cooling of S-A node. Upper row is control Lead II. When thermode was perfused rapidly with 4° C. water, the pacemaker shifted to A-V node, rate 35 with arrhythmia—middle row. Warm perfusion (45° C.) accelerated rate to 160—lower row.

The possibility of thermal injury was considered and was excluded by (a) invariable return of the T wave to control form, (b) absence of electrocardiographic injury effects (QRS, STT), and (c) absence of gross pathologic lesions at necropsy.

The intracardiac thermode used in the intact animal or man may prove valuable experimentally and therapeutically.

SUMMARY

1. A special intracardiac thermode for use in the intact animal or man is described.

2. Results obtained in the dog by perfusion of warm and cold water through this thermode placed in the approximate region of the S-A node are summarized.

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COMPARISON OF HUGGINS' TESTS WITH SEDIMENTATION RATE, WELTMANN REACTION, AND THE BOLEN TEST IN CANCER

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IN MARCH, 1949, Huggins, Miller, and Jensen[1] reported two tests for the detection of cancer, based on defective thermal coagulation of serum proteins. We are reporting our results using their tests on eighty-nine patients of whom forty-nine had malignant neoplastic disease and comparing the results with the sedimentation rate, Weltmann reaction, and Bolen test[2] performed on blood samples drawn at the same time.

METHODS

Huggin's Tests[1, 3].—In the procedure for obtaining the "iodoacetate index," buffered diluted serum is placed in several test tubes containing varying amounts of iodoacetate; these are immersed in a boiling water bath for thirty minutes. The tube containing the greatest amount of iodoacetate in which coagulation takes place is noted; from this amount and the quantitative serum protein determination, a value is computed and called the iodoacetate index. Normally it is 9.0 or more.

In the second test the "least concentration of coagulable protein" is determined by placing test tubes containing serial dilutions of serum in a boiling water bath for thirty minutes and noting the tube containing the greatest dilution in which coagulation takes place. Normally the concentration of serum proteins which will coagulate under these conditions is less than 1.5 Gm. per 100 cubic centimeters. A detailed description of these tests may be found in the original publication.[1]

The only modification made of Huggins' procedure was arbitrarily to use an N. P. N. value of 35 mg. per 100 c.c. in corrections made for serum protein determinations; if the N. P. N. was suspected or known to be high, actual values were determined.

To check our technical performance, repeat tests on three samples drawn on the same day were done and almost identical results obtained.

Sedimentation Rate and Weltmann Band.—For the erythrocyte sedimentation rate determinations the method of Wintrobe and Landsberg[4] was used. In the Weltmann reaction, the coagulability of diluted serum in decreasing concentrations of calcium chloride is noted. The modification recommended by Levinson and MacFate[5] was used.

Bolen Test.—This test is based on the pattern seen in a drop of blood allowed to dry on a clean glass slide. The blood is obtained by finger puncture.[2] In this series Bolen tests were performed on only fifty patients.

RESULTS

In Table I are given our findings on forty-nine cancer patients, along with diagnoses. In Table II are shown the number of positive tests per total determinations; percentages have been computed for comparison.

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TABLE I. HUGGINS' AND OTHER TESTS COMPARED IN CANCER

(False negative tests in italics. Diagnoses marked with asterisk (*) clinically proved; all others had histologic confirmation)

TEST	SEX	HUGGINS' TEST IAI 9.0+	HUGGINS' TEST LCCP 1.499–	SED. RATE M 10– F 20–	WELTMANN 5+	BOLEN NEG.	DIAGNOSES NORMAL VALUES
							Gastrointestinal Tract
1	M	5.97	1.989	36	4	*Neg.*	Squamous ca. tongue
2	M	8.0	2.58	*8*	2		Squamous ca. tongue
3	M	8.0	2.025	38	1–	*Neg.*	Squamous ca. tongue
4	M	7.08	2.013	12	2	*Neg.*	Adenoca. stomach
5	M	6.44	1.677	*6*	2		Ca. simplex stomach
6	F	8.25	1.635	24	*5*		Adenoca. stomach
7	M	*12.0*	1.54	*8*	*6*		Adenoca. stomach
8	F	8.33	1.901	21	3	Pos.	Squamous ca. rectum
9	M	8.93	1.772	*10*	*5*	*Neg.*	Adenoca. sigmoid with polyp
10	F	7.75	2.040	26	1–		Adenoca. sigmoid
11	F	5.57–	3.913+	*16*	1–	Dbt.	Adenoca. rectum
12	M	7.5	2.098	40	1	Pos.	Adenoca. colon (R)
13	F	6.5	2.19	32	1	Pos.	Adenoca. sigmoid, metas. to liver*
14	F	5.91–	2.436	*8*	3		Ca. rectum*
15	M	*9.0*	1.76	*4*	1–		Adenoca. colon
16	M	*16.2+*	*1.202*	43	1		Adenoca. rectum
17	F	*10.0*	1.635	*19*	2		Ca. sigmoid*
							Respiratory Tract
18	M	8.95	1.809	*6*	1–		Squamous ca. sinuses
19	M	5.63	2.108	*5*	1–		Squamous ca. larynx
20	M	7.96	1.809	35	*6*	Pos.	Squamous ca. larynx
21	F	*9.1*	1.57	36	1	*Neg.*	Squamous ca. larynx
22	M	8.45	1.789	40	4	Pos.	Ca. lung
23	M	4.75	2.122	35	2		Ca. lung
24	M	7.79–	2.662	36	1–	*Neg.*	Ca. rt. pleura and lung
25	F	8.02	1.884	36	4		Ca. bronchus
							Genitourinary Tract
26	M	7.2	2.25	12	3	Dbt.	Adenoca. kidney
27	F	*12.0*	*1.25*	41	5	Dbt.	Transitional cell, renal pelvis
28	F	8.73	1.923	38	*6*		Transitional cell, renal pelvis (R)
29	F	5.97	2.110	*5*	3		Papillary ca. bladder*
30	M	*10.94*	1.809	17	3		Transitional cell, ca. bladder
31	F	6.49	1.662	38	1–		Adneoca. uterus
32	F	*10.47*	*1.031*	*19*	3	*Neg.*	Adenoca. uterus
33	F	6.36	1.867	*10*	1–		Adenoca. pelvis recurrent
34	F	6.5	2.313	54	3	*Neg.*	Adenoca. abdomen, ovary?
35	F	7.2	1.75	34	1–	Dbt.	Adenoca. ovary
36	M	6.05	2.351	23	2		Ca. prostate, x-ray metastases*
							Breast
37	F	8.42	*1.436*	*15*	1		Ca. breast
38	F	6.4	1.854	41	1		Scirrhous ca. breast
39	F	7.86	2.013	38	2	Dbt.	Ca. simplex breast
40	F	5.9	1.989	*10*	1		Localized intracystic breast
41	F	*9.6*	1.65	40	1–	Pos.	Ca. breast
42	F	*12.93*	*1.206*	28	*6*	*Neg.*	Adenoca. breast
43	F	*9.95*	1.507	30	*6*		Adenoca. breast (R)
44	F	*9.42*	1.782	36	1–		Adenoca. breast

(*Continued on following page.*)

TABLE I—CONT'D

TEST	SEX	HUGGINS' TEST IAI 9.0+	HUGGINS' TEST LCCP 1.499–	SED. RATE M 10– F 20–	WELTMANN 5+	BOLEN NEG.	DIAGNOSES NORMAL VALUES
							Skin
45	M	8.95	2.291	13	*5*	*Neg.*	Basal cell ear and scalp
46	M	*10.28*	*1.4*	*3*	*6*	*Neg.*	Basal cell, back
47	F	5.97	1.809	45	1–		Squamous ca. scalp, face
48	F	7.11	1.890	23	*6*		Squamous ca. skin
							Miscellaneous
49	M	*9.86*	*1.398*	36	3	*Neg.*	Melanoma, conjunctiva
50	M	7.61	2.127	16	4		Melanoma, conjunctiva (R)
51	F	7.24	2.236	30	1–		Fibrosarcoma thigh
52	M	5.97	1.989	38	*6*		Ca. retroperitoneal, origin?
53	F	8.85	2.196	36	*8*		Multiple myeloma
54	M	*11.58+*	1.548	30	*9*	Dbt.	Malignant lymphoma

(R), Repeated tests.
IAI, Iodoacetate index; LCCP, least concentration coagulable protein.
Dbt., Doubtful.

TABLE II. TRUE POSITIVE TESTS IN CANCER CASES

	HUGGINS' TEST IAI	HUGGINS' TEST LCCP	SED. RATE	WELTMANN	BOLEN
Number	39/54	47/54	38/54	41/54	13/25
Per cent	72	87	70	76	52

IAI, Iodoacetate index.
LCCP, Least concentration of coagulable protein.

In reviewing and comparing the results of different tests in cancer cases, a number of observations may be made. Although the percentages given permit a rough evaluation of sensitivity, there were individual examples of each test giving a true positive when others were negative. The "least concentration of coagulable protein" was more sensitive than the iodoacetate index, and in only one instance (Case 37, Table I) did it fail to indicate cancer when the iodoacetate index was positive.

We also think it to be noteworthy that the Huggins tests have been positive in two patients with localized or small cancers (Cases 9 and 40) and negative in one patient with a large fungating growth (Case 47).

In this series the Bolen test seldom detected a cancer when other tests failed to do so. In some patients the sedimentation rates were elevated when the Bolen tests were negative, apparently indicating that the two depend on different types of alterations of plasma components.

In Table III are given the number and percentages of false positive tests, i.e., positive tests in the absence of cancer. The percentage of "false positive" in nonmalignant disease was rather high (from 35 to 66 per cent) for all the tests in our hands.

It should be pointed out that a number of the patients giving false positive tests were very sick. They were included deliberately to subject the procedure to conditions in which false positives were most likely to occur. Very few false

TABLE III. NONMALIGNANT CASES, POSITIVE TESTS

NUMBER OF CASES	DIAGNOSIS	IAI	LCCP	SED. RATE	WELTMANN BAND	BOLEN
3	Tuberculosis	1	1	2	2	1/3
6	Syphilis	2	3	5	1	2/4
4	Chr. cyst. mastitis	2	3	3	2	1/2
2	Cirrhosis	0	0	2	0	2/2
24	Miscellaneous	9	13	14	11	4/15
39	Total	14	20	26	16	10/26
	Per cent	35	51	66	41	38
12	Normals (controls)	0	0	0	2	

Diagnoses in the miscellaneous group which gave false positive IAI tests were: uremia from arteriolar nephrosclerosis, prostatic hyperplasia, pregnancy at term with meningitis, actinomycosis, intestinal obstruction due to intussusception, osteoarthritis of spine, undiagnosed gastrointestinal hemorrhage (G. I. series negative), cholelithiasis and common duct obstruction, and upper lobe pulmonary fibrosis and bronchiectasis.

positives were seen in relatively healthy patients taken at random. It is to be noted that except for two positive Weltmann reactions, no false positives were obtained in twelve normal subjects.

"Correct results" (positive in cancer, negative in the absence of cancer) were computed for comparison and the following percentages obtained:

HUGGINS				
IAI	LCCP	SED. RATE	WELTMANN BAND	BOLEN
68%	68%	52%	67.5%	55%

Although not too much significance may be attached to percentile difference of this magnitude when based on ninety-three determinations, the Huggins tests and Weltmann reaction seem to be similar in accuracy and possess some advantage over the simpler sedimentation rate and Bolen test. It should be noted that the figures on the Bolen test are based on a smaller number of determinations, and we believe this number of tests may not be sufficient for a fair evaluation.

In Table IV are summarized a comparison of our findings with those of Huggins in the use of the iodoacetate index. We have not had as high a percentage of true positives in cancer cases as did Huggins, and we also have had a greater percentage of false positives. In looking over our records we are unable to explain our failures on the basis of type of case or advancement of disease.

TABLE IV. POSITIVE IODOACETATE INDICES

	HUGGINS' FINDINGS	OUR FINDINGS
Normal	0/100— 0%	0/12— 0%
Nonmalignant pathology	16/95 —16%	15/40—37%
Malignancies	85/88 —96%	36/50—72%

SUMMARY AND CONCLUSIONS

A detailed report has been given listing values obtained in Huggins' tests on eighty-nine patients along with results of erythrocyte sedimentation rate, Weltmann reaction, and the Bolen test.

The tests have been compared for sensitivity and accuracy in detecting cancer. The determination of "least concentration of coagulable protein" was most sensitive, but gave many false positive tests. The iodoacetate index though

less sensitive gave fewer false positives. In over-all accuracy the two Huggins tests and the Weltmann reaction were about the same (correct in about two-thirds of the determinations). It may be noteworthy that all three are based on thermal coagulation.

In conclusion, we believe that the Huggins tests are of value in cancer detection, but in our hands they did not prove much more advantageous than the less expensive Weltmann reaction. On the basis of the results we have been able to secure, it is thought that none of these tests is of much value in the differential diagnosis of cancer. However, although our results proved less accurate in diagnosis than did Huggins', we feel that further trials of this and similar procedures are worth while.

Grateful acknowledgment is made of the helpful advice given by Dr. E. Lawrence Keyes, Chairman, Cancer Control Committee, St. Louis University School of Medicine.

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VITAMIN B_{12} CONCENTRATE IN THE MAINTENANCE OF PERNICIOUS ANEMIA

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THE isolation of crystalline vitamin B_{12} has provided the clinician with another pure chemical substance for therapeutic use. The demonstration[1] of its presence in liver and liver extracts effective in treating pernicious anemia implied that it was responsible for the antipernicious anemia effects of liver extract. Clinical testing has substantiated this.

Most of the data on clinical results published thus far have involved short-term studies on patients in relapse, both hematologic and neurologic. Thus, it has been shown that vitamin B_{12} is effective in producing a satisfactory hematopoietic response comparable in every respect to that obtained with liver extracts.[2, 3, 4] In all studies thus far reported, the crystalline material has been effective also in controlling the neurologic and lingual complications of pernicious anemia.[5, 6, 7]

The short-term studies have indicated that between 0.75 and 1.0 μg of vitamin B_{12} produces about the same effect in pernicious anemia patients as 1 U.S.P. unit of liver extract.[3] More recently, it has been suggested that larger amounts of vitamin B_{12} are necessary to produce a maximum effect.[8]

The purpose of this study was to ascertain whether vitamin B_{12} was as fully effective as liver extracts in maintaining remission in patients with pernicious anemia over longer periods of time, and to attempt to establish the maintenance dose when treatment was given at intervals of three to four weeks as is customary in the maintenance treatment of pernicious anemia.

MATERIALS AND METHOD

The products tested were microbiologically produced vitamin B_{12} concentrates.* At the beginning of the study, a cruder preparation known as APF-60 (Lederle) was used. This material was a product of the growth of a nonmotile, rod-like bacterium isolated from chicken feces as described by Stokstad.[9] It was labeled in terms of antipernicious anemia units, this potency being determined by comparison of its animal protein factor activity for chicks with that of standard preparations of liver extract of known clinical potency. These determinations were made at the Lederle Laboratories under the direction of Dr. Thomas H. Jukes. This material was given to patients by intramuscular injection at intervals of three to four weeks in doses equivalent to 1 U.S.P. unit per day (e.g., 21 units every three weeks).

A second preparation with the trade name Normocytin (Lederle) was a more concentrated material derived from the same microorganism as APF-60. On the basis of a considerable number of chick assays, it was found to have a potency intermediate between 10 and 15 U.S.P. antipernicious anemia units per milliliter.[10] Microbiologic assays with

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*The vitamin B_{12} concentrates were supplied by Dr. Thomas H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

Lactobacillus leichmannii 313 indicated a vitamin B_{12} content of 11 μg per milliliter.[10] This material was also labeled in terms of U.S.P. antipernicious anemia units and was administered intramuscularly on the same basis as the APF-60.

The final preparation used, also bearing the trade name Normocytin (Lederle), was a highly purified, although not crystalline, material supplied as a crystal-clear, slightly pink-colored solution. This material was produced by the microorganism *Streptomyces aureofaciens* and was prepared by concentration of the pink fraction from the organism.[10] This pink fraction later was found to be vitamin B_{12b}.[11] The preparation had an indicated vitamin B_{12} content of 20 μg per milliliter when assayed with *Lactobacillus leichmannii* 313 as well as with chick assay. A similar result was obtained by reading its absorption in the Beckman spectrophotometer at 535 millimicrons.[10] This preparation was used during the last eight months of the study and was given by intramuscular injection at intervals of three to four weeks so that the daily dose was 1 microgram.

Thirty patients with addisonian pernicious anemia are included in this study. All have been observed for long periods of time in the medical outpatient department of the University Hospitals of Cleveland. The diagnosis had been established sometime previously by the presence of macrocytic anemia, megaloblastic bone marrow, and histamine-fast achlorhydria. All had responded in a characteristic manner to antipernicious anemia therapy. Most had had either lingual or neurologic symptoms and signs, or both, at one time or another but these were not prominent at the outset of the present study.

RESULTS

In comparing the results of treatment with liver extracts, pteroylglutamic acid and vitamin B_{12} concentrate, arbitary standards described in a previous publication[12] have been employed. A change in the erythrocyte count of 300,000 per cubic millimeter or less is not considered significant. A change of 300,000 to 500,000 per cubic millimeter is considered moderate, and a change of over 500,000 per cubic millimeter marked. All of the hematologic determinations were made by a single technician using uniform equipment. Hemoglobin determinations were made with the Duffy hemoglobinometer.[13]

The patients were treated from five to fourteen months with the vitamin B_{12} concentrates. Twenty of the thirty patients were treated for twelve or more months.

Twenty-three of the thirty patients had been treated previously for a period of at least one year with crude liver extract containing 2 U.S.P. units per milliliter. Twenty-one patients maintained erythrocyte and hemoglobin levels considered to be satisfactory (Table I) without occurrence of lingual or neurologic relapse. These patients received an average of 10 U.S.P. units (5 ml.) of liver extract given intramuscularly at intervals of four weeks. It is recognized that this represents less than the average of 1 U.S.P. unit daily considered optimal. While we do not recommend this regimen as a standard form of therapy, it has been our experience that when crude liver extracts are used, the unit dosage referred to is effective in maintaining adequate hematologic and neurologic remission. The blood values in Table I are averages of all erythrocyte and hemoglobin determinations during the last year on intramuscular liver therapy.

Fifteen of the patients who had been treated for at least one year with crude liver extract were treated during the subsequent one or two years with

TABLE I. COMPARISON BETWEEN INTRAMUSCULAR TREATMENT WITH VITAMIN B_{12} CONCENTRATE AND PREVIOUS TREATMENT WITH LIVER EXTRACT AND

TABLE I. COMPARISON BETWEEN INTRAMUSCULAR TREATMENT WITH VITAMIN B_{12} CONCENTRATE AND PREVIOUS TREATMENT WITH LIVER EXTRACT AND PTEROYLGLUTAMIC ACID

CASE	AGE (YR.)	SEX	RACE	AV. AFTER LIVER EXTRACT*		AV. AFTER FOLIC ACID†		DIFFERENCE L.E. VS PGA		TIME ON NORMOCYTIN (MO.)	DOSE (μg)	TIME BETWEEN DOSES (WK.)	AV. AFTER NORMOCYTIN		DIFFERENCE NORMOCYTIN VS L.E.		DIFFERENCE PGA VS NORMOCYTIN	
				R.B.C. ($\times 10^6$)	Hgb. (GM.)	R.B.C. ($\times 10^6$)	Hgb. (GM.)	R.B.C. ($\times 10^6$)	Hgb. (GM.)				R.B.C. ($\times 10^6$)	Hgb. (GM.)	R.B.C. ($\times 10^6$)	Hgb. (GM.)	R.B.C. ($\times 10^6$)	Hgb. (GM.)
1‡	53	F	W	4.82	13.5	--	--	--	--	13	40	4	4.24	12.9	−0.58	−0.6	--	--
2	62	M	W	3.96	13.4	3.02	11.3	−0.74	−2.1	12	20	3	4.23	12.9	+0.27	−0.5	+1.21	+1.6
3	74	M	W	--	--	--	--	--	--	6	20	3	3.84	12.8	--	--	--	--
4	59	F	W	4.64	13.1	--	--	--	--	7	20	3	3.78	13.3	−0.86	+0.2	--	--
6	76	M	W	4.12	12.7	--	--	--	--	13	20	3	3.29	12.2	−0.83	−0.5	--	--
8	54	F	W	4.51	13.9	5.02	14.05	+0.51	+0.6	14	20	3	3.71	12.5	−0.80	−1.4	−1.31	−1.55
9	59	M	N	4.79	13.3	5.19	13.7	+0.40	+0.4	12	20	3	4.96	13.1	+0.17	−0.2	−0.23	−0.6
10	73	F	W	4.48	13.8	3.94	12.2	−0.54	−1.6	12	20	3	3.50	12.3	−0.98	−1.5	−0.44	+0.1
11	54	F	N	4.53	14.0	5.02	14.4	+0.49	+0.4	11	20	3	3.91	12.9	−0.62	−1.1	−1.11	−1.5
12	67	M	W	--	--	5.06	15.6	--	--	13	20	3-4	4.09	12.9	--	--	−0.97	−2.7
13	61	M	N	4.19	12.9	4.23	12.9	+0.04	0.0	14	20	3	3.82	13.3	−0.37	+0.4	−0.41	+0.4
14	77	F	W	--	--	3.99	13.4	--	--	12	20	3	3.37	12.5	--	--	−0.62	−0.9
15	51	F	W	4.03	12.5	4.06	12.1	+0.03	−0.4	12	20	3	3.57	11.6	−0.46	−0.9	−0.63	−0.5
16	54	F	W	--	--	--	--	--	--	8	20	3	3.42	12.2	--	--	--	--
17	54	F	W	4.65	13.6	5.01	13.6	+0.36	0.0	10	20	3	4.20	13.6	−0.45	0.0	−0.82	0.0
18	59	F	W	4.20	13.9	4.70	15.0	+0.50	+1.1	13	20	3	4.01	12.2	−0.19	−1.7	−0.69	−2.8
19	67	M	W	4.77	13.2	--	--	--	--	7	20	3	3.96	13.6	−0.81	−0.4	--	--
21	45	F	W	4.30	10.5	--	--	--	--	8	20	3	3.81	12.7	−0.49	+2.2	--	--
22	60	F	N	4.47	12.9	--	--	--	--	12	20	3	3.67	12.7	−0.80	−0.2	--	--
23	87	M	W	3.60	12.7	3.9	12.8	+0.30	+0.1	12	20	3	2.96	10.5	−0.64	−2.2	−0.94	−2.3
24	68	F	W	4.61	13.2	--	--	--	--	14	20	3	3.72	12.8	−0.89	−0.4	--	--
25	57	M	W	4.59	12.8	--	--	--	--	7	20	3-4	4.10	13.1	−0.49	+0.3	--	--
26	77	M	W	4.15	13.9	4.78	16.1	+0.63	+2.2	13	20	3	4.65	15.5	+0.50	+1.4	−0.13	−0.6
27	65	M	W	4.62	13.4	4.82	14.5	+0.20	+1.1	14	20	3	3.89	13.3	−0.73	−0.1	−0.93	−1.2
28	83	M	W	4.48	13.4	4.88	14.0	+0.40	+0.6	13	20	3	3.85	12.8	−0.63	−0.6	−1.03	−1.2
29	75	M	W	4.38	14.0	4.85	14.7	+0.47	+0.7	13	20	3	3.87	13.4	−0.51	−0.6	−0.98	−1.3
30	52	F	W	--	--	--	--	--	--	9	20	3	4.00	13.3	--	--	--	--
32	54	F	W	--	--	5.59	16.1	--	--	12	20	3	3.60	12.3	--	--	−1.99	−3.8
33	79	F	W	4.56	14.0	4.81	14.9	+0.25	+0.9	5	20	4	3.66	12.3	−0.90	−1.7	−1.15	−2.6
35	65	F	W	--	--	--	--	--	--	14	20	3	4.25	12.8	--	--	--	--

*Average of counts over period of last year on intramuscular liver extract therapy.
†Average of three blood counts at end of one- or two-year period on folic acid.
‡Case 1 received intramuscular liver extract and daily oral folic acid simultaneously.

folic acid either orally in doses of 10 mg. daily or in doses of 50 to 150 mg. at intervals of two to six weeks parenterally. As noted previously,[12] intermittent parenteral therapy with folic acid was not effective in maintaining normal blood values, so that during the second year of the folic acid study all patients received 10 mg. daily oral doses.

A comparison of the blood values during the year on liver extract therapy with those on oral folic acid (Table I) shows that in only two instances was there a decrease in the erythrocyte count and hemoglobin during folic acid medication, although in each instance the decrease in erythrocyte count was greater than 500,000 cells per cubic millimeter, a significant lowering. The thirteen remaining patients showed an increase in blood values on folic acid. Five patients showed insignificant increase in the erythrocyte counts (30,000 to 300,000 per cubic millimeter); six patients showed moderate increase (300,000 to 500,000 per cubic millimeter), and two showed marked increase (630,000 and 510,000 per cubic millimeter respectively). Of these fifteen patients, two developed neurologic relapse and one developed glossitis while on folic acid therapy. The fact that higher erythrocyte and hemoglobin levels were obtained after folic acid therapy as compared with values on liver extract therapy indicates to us that treatment with liver extract therapy had not produced optimal results, because of an insufficient dose.

The thirty patients presented here were treated with vitamin B_{12} concentrate in the dosages and at the intervals previously described for durations varying from five to fourteen months. There were twenty patients whose erythrocyte counts were less than 4,000,000 per cubic millimeter and ten patients whose erythrocyte counts were over 4,000,000 per cubic millimeter at the end of this term of therapy (Table II). Moreover, there were only two patients whose erythrocyte counts were over 4.3 million per cubic millimeter at the end of the period, and five patients showed levels of 3.5 million per cubic millimeter or less at the end of therapy (Table I).

Nine patients had less than 12.5 Gm. of hemoglobin per 100 ml. as compared with twenty-one patients with hemoglobin of 12.5 Gm. or more per 100 ml. (Table II).

TABLE II. BLOOD VALUES AT END OF PERIOD OF TREATMENT WITH VITAMIN B_{12} CONCENTRATE

ERYTHROCYTES (PER C.MM.)	NUMBER OF PATIENTS
Less than 4,000,000	20
More than 4,000,000	10
HEMOGLOBIN (GM. PER 100 ML.)	**NUMBER OF PATIENTS**
Less than 12.5 Gm.	9
More than 12.5 Gm.	21

In the group of thirty patients receiving vitamin B_{12} concentrate, twenty-three patients had received crude liver extract therapy previously. A comparison of the blood values after vitamin B_{12} concentrate therapy with those obtained during liver extract therapy reveals (Table III) that twenty of the twenty-three patients had a decrease in the erythrocyte count, fourteen showing

a marked decrease (over 500,000 per cubic millimeter), five a moderate decrease (300,000 to 500,000 per cubic millimeter), and one an insignificant decrease (190,000 per cubic millimeter). Of three patients whose blood values improved during treatment with vitamin B_{12} concentrate, two patients showed only insignificant increases (170,000 and 270,000 per cubic millimeter) while the third had a moderate increase of 500,000 erythrocytes per cubic millimeter.

TABLE III. CHANGES IN ERYTHROCYTE COUNTS AFTER TREATMENT WITH VITAMIN B_{12} CONCENTRATE COMPARED WITH LIVER EXTRACT

CHANGE (PER C.MM.)	LOSS ON VITAMIN B_{12} CONCENTRATE	GAIN ON VITAMIN B_{12} CONCENTRATE
0-300,000	1	2
300-500,000	5	1
Over 500,000	14	0
Total	20	3

Eighteen of the thirty patients treated with vitamin B_{12} concentrate had been treated with oral folic acid during the year previous to this study. A comparison of the blood values after vitamin B_{12} concentrate with those during folic acid therapy (Table IV) reveals that seventeen of the eighteen patients had a decrease in erythrocyte count after vitamin B_{12} concentrate therapy. Five patients showed a decrease of over 1,000,000 erythrocytes per cubic millimeter, eight patients a decrease of 500,000 to 1,000,000 per cubic millimeter, two a decrease of 300,000 to 500,000 per cubic millimeter, and two a slight decrease of 230,000 and 130,000 per cubic millimeter respectively. In one patient the erythrocyte count increased 1.2 million per cubic millimeter. Thus, thirteen patients had a marked decrease, two patients a moderate decrease, and two an insignificant decrease in erythrocyte count as compared with blood values during previous folic acid therapy. Only one patient had a marked increase in erythrocyte count.

TABLE IV. CHANGES IN ERYTHROCYTE COUNT AFTER TREATMENT WITH VITAMIN B_{12} CONCENTRATE COMPARED WITH PGA

CHANGE (PER C.MM.)	LOSS ON VITAMIN B_{12} CONCENTRATE	GAIN ON VITAMIN B_{12} CONCENTRATE
0-300,000	2	0
300-500,000	2	0
Over 500,000	13	1
Total	17	1

CLINICAL OBSERVATIONS

During the course of treatment with vitamin B_{12} concentrate, all patients maintained a sense of well-being. There were no symptoms of weakness or undue fatigability despite erythrocyte counts of less than 4,000,000 per cubic millimeter in twenty of the thirty patients. This discrepancy between decline in erythrocyte count and lack of symptoms was particularly noteworthy in the five patients whose erythrocyte count dropped a million or more per cubic millimeter.

No patient developed signs or symptoms of neurologic relapse, nor did any patient with neurologic lesions show progression of these lesions. One patient (Case 30) was in neurologic relapse at the onset of treatment with complaint of numbness and tingling in her hands and feet associated with ataxia and complete loss of vibratory sensation in the legs. These abnormalities disappeared progressively after vitamin B_{12} concentrate therapy so that at the end of eight months the only residual symptom was minimal paresthesia of the hands, while ataxia disappeared and vibratory sensation returned to normal. This patient supports previous studies by others which indicate that vitamin B_{12} is effective in controlling neurologic relapse. This concept is further supported to the extent that no neurologic lesions appeared during treatment with vitamin B_{12} concentrate.

No patient developed any lingual signs or symptoms. No lingual complaints were present in this group at institution of treatment with vitamin B_{12} concentrate, with the exception of one patient (Case 23) who developed a beefy-red tongue while on folic acid therapy and responded satisfactorily to therapy with APF-60.

Significant toxic manifestations were not associated with the administration of the vitamin B_{12} concentrate. A few patients complained of mild transient pain and burning immediately after intramuscular (gluteus maximus) injection. The soreness which persists one or two days after liver extract injection was noticeably absent. In a group of patients of this size, treated over a period of this duration, it is inevitable that introduction of injected material into a vein occurred inadvertently at times. When this occurs with liver extract, patients frequently complain of a metallic taste and develop chills and fever. This did not occur in this group of patients. The only reactions observed were with the less purified APF-60. This material caused reactions on one occasion in each of two patients manifested by nausea, vomiting, perspiration, and giddiness and relieved by Adrenalin. No reactions occurred with the more purified Normocytin.

Several patients who were sensitive to liver extract were treated with the vitamin B_{12} concentrate. This sensitivity had been manifested by some or all of the following: urticaria, chronic dermatitis, chills and fever, prostration, and shock. These patients tolerated treatment with vitamin B_{12} concentrate without any manifestation of sensitivity whatsoever.

DISCUSSION

It is apparent that vitamin B_{12} concentrate was not effective in maintaining satisfactory blood levels in patients with pernicious anemia in the amounts used. The dose of 1 μg daily was chosen because previous reports in more acute experiments had indicated that 0.75 to 1 μg of vitamin B_{12} was equivalent in its effect to 1 U.S.P. antipernicious anemia unit of liver extract.[3] However, the more recent study of Jones and associates[8] indicated that the maximal rate of erythropoiesis would probably require more than 1 μg of vitamin B_{12} per day. Results obtained in our group of patients confirm this impression. Furthermore, even if 1 μg of vitamin B_{12} administered daily to patients in

relapse does regularly evoke a maximal response, it does not necessarily follow that remission can be maintained by injections at longer intervals of doses which average 1 μg daily. Since 1 μg of vitamin B_{12} concentrate daily administered at regular intervals of three to four weeks has not maintained satisfactory erythropoiesis, larger average daily doses of three times the amount are now being given to the same patients to determine if the failure of adequate response was due solely to suboptimal dosage. Continued observation of this group of patients will establish this point.

There are other possible reasons why the vitamin B_{12} concentrate used here was not effective in maintaining optimal erythrocyte levels:

(a) Although in acute experiments vitamin B_{12} has appeared to be equivalent in all respects to purified liver extracts, it is possible that liver extracts contain supplementary substances which augment the effect of vitamin B_{12}.

(b) The vitamin B_{12} content of the material used was regularly determined by microbiologic assay, although other assay methods were also employed. It is possible, although it seems unlikely, that the assay methods indicated the presence of larger amounts of vitamin B_{12} than were actually present.

(c) At least three forms of the vitamin, namely vitamin B_{12}, B_{12a}, [14] and B_{12b},[11] have been recognized to date. Even some crystalline preparations contain a mixture of B_{12} and B_{12b}. Preliminary studies[15] suggest that crystalline preparations of pure vitamin B_{12} and B_{12b} are equal in potency as tested in patients with pernicous anemia, as a growth factor for animals, and in microbiologic assays. It is possible, however, that vitamin B_{12} and B_{12b} do not have equal potencies for patients with pernicious anemia. Since differently prepared concentrates contain different proportions of B_{12} and B_{12b} (and probably B_{12a}), it is possible that, although the microgram content of different preparations is identical, the clinical potency may vary. The preparations derived from *Streptomyces aureofaciens* used in this study contained about 80 per cent B_{12b} and 20 per cent B_{12}.[10]

Of the possibilities considered here, the first presented, namely that an average of 1 μg daily is not sufficient dosage, seems the most probable to the investigators.

The inability of folic acid to prevent or control neurologic relapse has been well demonstrated. No neurologic or lingual relapse developed in this group of patients even though blood levels were not optimal. This is further evidence that vitamin B_{12} concentrate is effective in controlling the lingual and neurologic features of pernicious anemia. In addition, one patient who had evident neurologic manifestations at the time of the institution of therapy, showed marked improvement after treatment with vitamin B_{12} concentrate.

Vitamin B_{12} concentrate (Lederle) had no significant toxic effects.

Patients with sensitivity to liver extract can be treated with vitamin B_{12} with complete freedom from any manifestations of sensitivity.

SUMMARY

Thirty patients with pernicious anemia were treated with vitamin B_{12} concentrate for periods of five to fourteen months.

Vitamin B_{12} concentrate in the dose of 1 μg daily given by intramuscular injection every three to four weeks did not maintain optimal levels. Twenty of thirty patients had erythrocyte counts under four million at the end of the treatment period. Of twenty-six patients previously treated with folic acid or liver extracts, seventeen showed erythrocyte decrease of over 500,000 per cubic millimeter during treatment with vitamin B_{12} concentrate.

Reasons for failure to maintain optimal blood levels are discussed.

It seems likely that inadequate dosage is the best explanation for failure of adequate hematopoiesis although this is not proved.

No neurologic or lingual relapse developed in any patient. One patient had marked improvement of neurologic symptoms with therapy.

There was notable lack of toxic or allergic manifestations.

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APLASTIC CRISIS IN SICKLE CELL ANEMIA

A Study of Its Mechanism and Its Relationship to Other Types of Hemolytic Crises

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HEREDITARY spherocytosis and sickle cell anemia are the two chronic hemolytic syndromes in which the phenomenon of a "hemolytic crisis" may be encountered. Clinically the crisis manifests itself by a precipitous fall of the hemoglobin and erythrocyte levels, and by such constitutional symptoms as fever, chills, malaise, nausea, and vomiting. In sickle cell anemia, severe pain around the joints or in the abdomen is often present, at times simulating rheumatic fever or acute surgical conditions.

In hereditary spherocytosis the crisis is a rare event. It has been noted, however, that it does occur either simultaneously or in rapid succession in several members of the same family.[1, 6] These observations have led to the assumption that an extrinsic, possibly infective factor, may induce the crisis. In sickle cell anemia, hemolytic crises are frequently seen. In many patients they develop periodically, often at relatively constant intervals, and are apparently unrelated to any external influence.[7]

Recently the dynamic aspects of the crisis in hereditary spherocytosis have received intensive study. Several observers[3, 5, 6] have noted that the rapid fall in hemoglobin and red cell count is associated, at least in some instances, with reticulocytopenia as well as granulo- and thrombocytopenia. In these instances serial examinations of the marrow at the height of the crisis have revealed an "erythrocytic aplasia"[5, 8] which has also been interpreted as a maturation arrest at the proerythroblast level.[6] In the recovery phase, with or without splenectomy, the marrow reverts again to a hyperplasia of the erythropoietic tissue with the resulting release of a great number of reticulocytes.

In order to understand the crisis it is necessary to correlate these marrow findings with the fate of the spherocytes in the peripheral blood. The spherocytes in familial hemolytic jaundice are selectively removed by the spleen. The average survival time of these abnormal cells in the circulation is only fifteen to twenty days,[9, 10] which is about one-sixth the longevity of the normal erythrocytes.[11]

According to Owren[5] the mechanism responsible for the crisis is primarily the cessation of the production and release of the erythrocytes. The rapid removal of the spherocytes from the peripheral blood without any replacement

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from the marrow can account for the fall of the erythrocyte level. No additional splenic hyperhemolysis, as considered by other workers,[6, 12] need be assumed to explain the crisis which consequently should be designated "aplastic" rather than "hemolytic."[5]

In sickle cell anemia the mechanism causing the periodic crises is still obscure. Examination of the marrow regularly shows erythrocytic hyperplasia together with marked reticulocytosis. Thus the pathogenesis of the "hyperplastic" crisis in this disorder appears to be quite different from the "aplastic" type encountered in spherocytic anemia. However, it has been established recently that the life span of the erythrocyte in sickle cell anemia is also considerably shortened.[13, 14] In view of the possibly infective factor inducing the "aplastic" crisis in hereditary spherocytosis, one might expect to find a similar mechanism occasionally operating in sickle cell anemia. Such an observation is reported in this paper. The transitory aplasia of the erythropoietic tissue developed during the course of a clinically demonstrable virus pneumonia. Studies of the survival time of the patient's red cells were done in an effort to determine the hematologic mechanisms involved.

REPORT OF CASE

M. W., a 9-year-old Negro boy, known for several years to have sickle cell anemia, was admitted to the Sarah Morris Hospital with complaints of anorexia, fever, lethargy, generalized aching, and cough. The patient had been listless and had lost his appetite for about two weeks. Three days prior to admission he developed a temperature of 104° F., with the signs of an upper respiratory infection. The patient's 11-year-old sister, also suffering from sickle cell anemia, was admitted to another hospital with similar complaints on the same day.

The past history revealed that the patient had had mumps and pertussis at the age of 6 and 9 months respectively. For many years he had experienced attacks of migratory joint pains and periodic bouts of abdominal pains accompained by a severe anemia (Hgb. 7.8 Gm. [47 per cent], R.B.C. 3.0 million, and a positive sickle cell test).

Physical examination revealed a well-developed child who appeared moderately ill. Temperature 104° F.; pulse 120; respirations 28. The sclerae were slightly icteric. There was a mucopurulent discharge from the nose. The pharynx was injected. The posterior cervical lymphnodes were moderately enlarged. Lungs were clear. The left border of the heart was 2 cm. outside the nipple line. There was a loud systolic murmur at the apex. The liver edge extended two fingers below the costal margin in the midclavicular line and the tip of the spleen was palpable. The abdomen was soft and nontender. No joint changes or neurologic abnormalities were present.

A chest plate taken on the first day of admission showed a moderate infiltration in the first and second right interspaces (pneumonitis) and left hilar calcification. The cardiac contour was globular, the heart being slightly enlarged in its transverse diameter. An electrocardiogram (Dr. L. Katz) revealed: low T_2, diphasic T_3, and inverted T in V_2, V_4, and V_5. S-T segment depressed in Leads II and III, and in V_5. P_1 and P_3 were prominent.

Laboratory Data.—Urine: albumin and sugar absent, urobilinogen increased. Kahn: 3 plus positive. Blood: Hgb. 8.6 Gm. (55 per cent), R.B.C. 2.8 million, W.B.C. 3,650. Film: no polychromasia of the red cells, moderate number of target cells and sickle cells. The reticulocyte count was 0.2 per cent. Differential count: neutrophils, segmented 37 per cent, nonsegmented 8 per cent; basophilic granulocytes 1 per cent; lymphocytes 38 per cent; monocytes 16 per cent. No toxic granulation of the neutrophils. Numerous platelets on the film.

The unusual observation of such a low reticulocyte count in a case of active sickle cell anemia was explained by the state of the marrow. Sternal puncture was performed on the second hospital day. Although the marrow was quite cellular there was an extreme depression of red cell production. A few proerythroblasts and basophilic erythroblasts but no giant forms of these cell types were seen (Table II). The white cell series showed a slight shift to the left and a moderate increase of the plasma cells and histiocytes (reticulum cells).

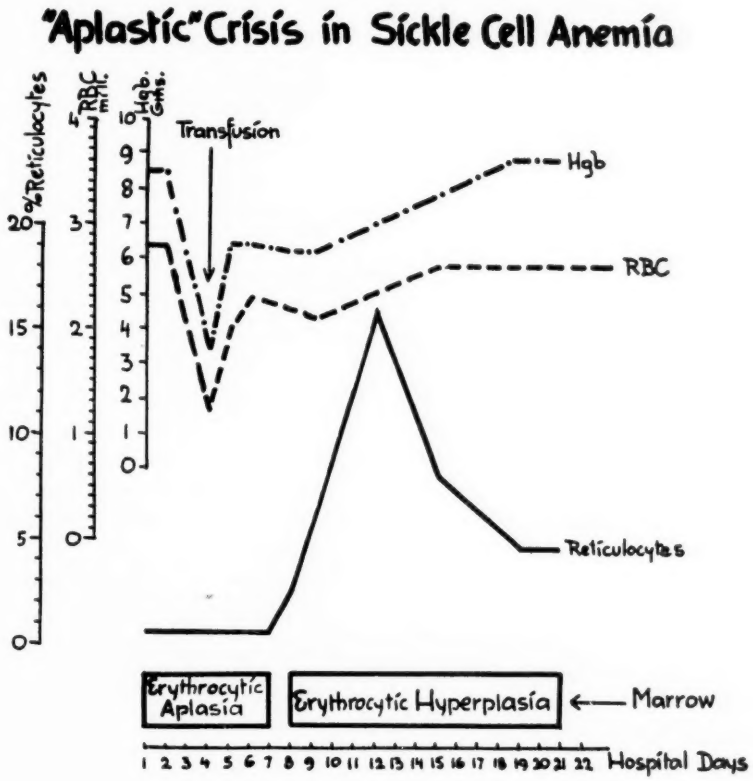

Fig. 1.

Further laboratory studies on the second hospital day revealed the following findings. *Serology* (Dr. Milzer): No cold agglutinins. Heterophile agglutination negative. Complement fixation tests for the psittacosis-pneumonitis group, influenza A and B, Q fever, and lymphocytic choriomeningitis were all negative. Agglutination for streptococcus MG negative. Mantoux test (O. T.) 1:1000 one plus positive in 48 hours. *Bacteriology:* Blood cultures, no growth. Throat culture, *Streptococcus viridans* and *Staphylococcus albus*. *Chemistry:* Serum bilirubin 1.4 mg. per cent, cholesterol 96 mg. per cent with 72 per cent esters, thymol turbidity and flocculation within normal range.

Course.—The patient's temperature became normal on the fourth hospital day without any antibiotic or chemotherapy, but it was noted that he was even more drowsy and listless than previously.

Hematologically a definite "crisis" developed. The hemoglobin value dropped from 8.6 to 3.5 Gm. and the red count from 2.8 to 1.2 million within seventy-two hours (Fig. 1). The reticulocytopenia in the peripheral blood continued. There was granulocytopenia but the differential count showed 18 per cent plasma cells (Table I). The platelet count was only very slightly depressed (230,000 against 300,000 normal, Dameshek's method[15]). A

TABLE I. WHITE CELL COUNTS, PLATELETS, AND DIFFERENTIAL COUNTS

HOSPITAL DAY	W.B.C.	PLATELETS (300,000 NORMAL)	DIFFERENTIAL COUNT (%)						
			SEGMENTED NEUTROPHILS	NON-SEGMENTED NEUTROPHILS	EOSINOPHILS	BASOPHILS	LYMPHOCYTES	MONOCYTES	PLASMA CELLS
1	3,650	Numerous on film	37	8	0	1	38	16	0
2	3,700	220,000	Not determined						
4	6,400	230,000	17	4	0	1	49	11	18
5	4,680	Numerous on film	34	5	2	0	31	10	18
8	10,200	Numerous on film	42	16	7	1	20	12	2
12	9,800	Numerous on film	60	2	2	1	23	11	1
15	10,700	Numerous on film	65	2	5	0	17	10	1

Coombs antiglobulin test[26] was negative. There was no noticeable increase of the jaundice and no dehydration. A transfusion of 250 c.c. packed red cells was given which increased the level of the hemoglobin to 6.5 Gm. and the red count to 2.2 million. As can be seen from Fig. 1, following the transfusion the patient's red count and hemoglobin did not significantly decrease further and later rose gradually to the levels found at admission.

On the ninth hospital day reticulocytes reappeared in the peripheral blood (5.6 per cent). Two days later the number of reticulated red cells amounted to 16 per cent (Fig. 1) and there were 13 nucleated oxyphilic red cells per 100 white cells. Marrow examination showed a complete reversal of the previous findings: very marked erythrocytic hyperplasia, the polychromatic normoblasts being the predominant cell (Table II). No increase in plasma cells was noticeable any longer.

The patient maintained the elevated reticulocyte count thereafter. During the course of the illness cold agglutinins (titer of 1:160) appeared and the formerly positive Kahn test turned negative. These latter findings confirmed the diagnosis of "atypical virus pneumonia." Several gastric washings were negative for acid-fast organisms. The electrocardiogram returned to a normal pattern. One month after admission, an x-ray examination of the chest showed partial resolution of the infiltrate and after two months there was complete resolution.

A hematologic examination seven months later showed a hemoglobin of 8.0 Gm. (50 per cent) and a red count of 2.6 million with a reticulocyte count of 9.6 per cent. Study of the marrow revealed an erythrocytic hyperplasia but of lesser degree than seen in the recovery phase from the crisis (Table II). The patient seems well adapted to the chronic hemolytic anemia.

TABLE II. BONE MARROW STUDIES; DIFFERENTIAL COUNT OF NUCLEATED RED BLOOD CELLS

BONE MARROW PATTERN	ERYTHROGRANULOCYTIC RATIO	PRONORMOBLASTS	BASOPHILIC NORMOBLASTS	POLYCHROMATOPHILIC NORMOBLASTS	ORTHOCHROMATOPHILIC NORMOBLASTS
		PER 100 MARROW CELLS			
Aplastic phase	1:15	3.2	2.8	0.25	0.25
Hyperplastic phase	2.6:1	2.2	16.0	45.0	9.2
Chronic state (7 mo. after recovery from crisis)	1.25:1	3.0	9.5	34.5	8.5

The patient's sister, who was admitted with similar complaints to another hospital the same day, apparently did not show the signs of an "aplastic crisis," since nucleated red cells and polychromatophilia were found in the peripheral blood during her illness. Her

hemoglobin value was 5.5 Gm. and the red count 2.0 million. X-ray examination of the lungs was negative, but a false positive Kahn test was observed in this case too. Unfortunately no bone marrow studies were done.

In summary then, a patient with known sickle cell anemia, developed an erythrocytic aplasia of the marrow associated with reticulocytopenia, leucopenia, and slight thrombocytopenia in the peripheral blood during the course of a clinically demonstrable virus pneumonia. A "crisis" followed in which the patient's red count dropped from 2.6 to 1.2 million and the hemoglobin from 8.6 to 3.5 Gm. within three days. Immediately after one blood transfusion the red cell count increased about 1 million per cubic millimeter and continued at this level, although the reticulocytopenia persisted for several days. Later, reticulocytes reappeared in the blood in large numbers and the marrow then showed a very marked erythrocytic hyperplasia.

STUDIES ON THE SURVIVAL TIME OF THE PATIENT'S ERYTHROCYTES

It was stated that according to Owren's hypothesis[5] the mechanism of the "aplastic" crisis in hereditary spherocytosis consists of the combination of (1) an absent red cell production and release and (2) an unaltered rapid elimination of the spherocytes from the peripheral blood. Since no increase of erythrocyte destruction above the precrisis level is considered to be present, it follows that the diminution of the erythrocyte value during the crisis will depend solely on the speed with which the peripheral cells are removed from the circulation. From the standpoint of this concept, it may then further be assumed that determination of the survival time of the patient's erythrocytes some time following his recovery may reveal a reasonably good correlation between the rates of hemolysis during and after the crisis. This assumption implies that the composition of the red cell populations during and after the crisis in the same patient is very similar—a postulation which may or may not necessarily be correct. However, if the rates of red cell disappearance should correspond in both instances, such a finding would support Owren's hypothesis. Consequently, if in our patient with sickle cell anemia a true "aplastic" crisis existed, a study of the life span of the erythrocytes should demonstrate an extremely rapid elimination, inasmuch as during the crisis 57 per cent of the red cells disappeared from the circulation within three days.

Seven months after discharge from the hospital, when the patient had completely recovered from his attack of pneumonia, his red cells were transfused into a 4-year-old boy who was also afflicted with sickle cell anemia. This recipient was selected in order to create an environment for the transfused cells similar to that present in the patient's own circulation. The methods used in these survival time studies (a modified Ashby technique of differential agglutination) have been described in detail in previous communications.[13, 16]

Fig. 2 depicts the results of these experiments. Graph *1* visualizes the disappearance rate of transfused normal red cells, which was first determined as a control to rule out any possibly existing extrinsic mechanism of erythrocyte disintegration in this recipient. A characteristic straight "line of decay" with a survival time of 120 days was obtained. Graph *2* shows the removal of the transfused sickle cell anemia cells obtained from our patient. As can be seen, 47 per cent of them were eliminated within three days and 60 per cent within eight days; the remaining cells disintegrated more slowly but after twenty-eight days were no longer demonstrable.

These results, therefore, seem to support the concept of Owren. Although 57 per cent of the circulating erythrocytes disappeared within the first three days during the crisis and only 47 per cent in our transfusion experiment, the discrepancy of 10 per cent is most likely explained by the inherent errors of the methods used.

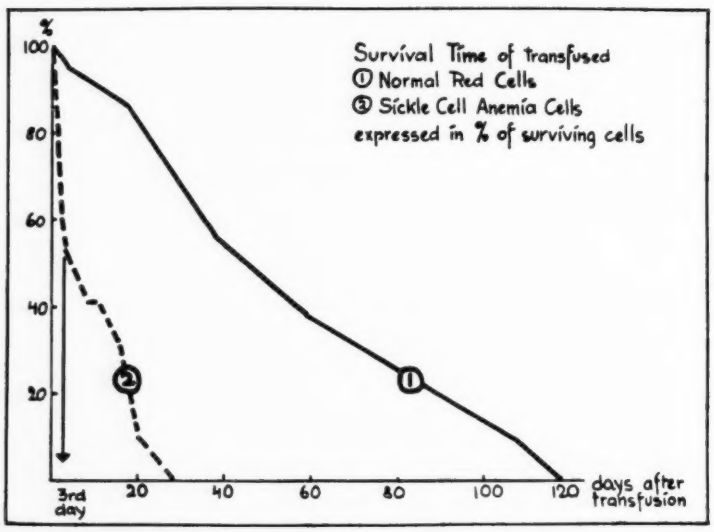

Fig. 2.—Survival time of the patient's erythrocytes and of normal cells in the same recipient.

Transfused normal erythrocytes: 1.1 million = 100 per cent.
Transfused anemia cells: 320,000 = 100 per cent.

It should be pointed out that the red cell population of this patient is obviously not a homogenous one, since the elimination curve clearly indicates that a certain proportion of his erythrocytes is more readily hemolyzed than are others. This feature is quite characteristic and in agreement with the earlier findings of Singer and associates[13] and Callender and co-workers[14] who observed in their studies with transfused sickle cell anemia cells a rapid initial elimination of cells, varying in degree from case to case; later on the speed of hemolysis decreased and some portions of the remaining erythrocytes were destroyed at a relatively much slower rate.[14]

Fig. 3 visualizes the changes in the survival time graph which can be noted when a mixture of two erythrocyte populations with different average life spans are transfused into the same recipient. Such a mixture may be obtained by adding a proportion of sickle cell anemia cells to normal erythrocytes. By proper selection of the types of red cells used (OM anemia and ON normal cells transfused into a BM recipient), the elimination of the two parts of the mixture as well as their sum total can be followed separately by differential agglutination. As can be seen from Fig. 3, the disappearance curve of the total cell population shows a definite "shift to the left" because of the presence of the short-lived sickle cell anemia cells.* This shift occurred, even though for technical reasons

*The cells were obtained from another patient with sickle cell anemia showing a hemoglobin of 8.7 Gm. and a red cell count of 2.9 million.

the ratio of anemia to normal cells was only 1:3 in our experiment. It is obvious that with a different ratio more in favor of the anemia cells, this shift to the left would be much more pronounced and a curve more closely resembling the initial slope of graph 2 in Fig. 2 would then be obtained.

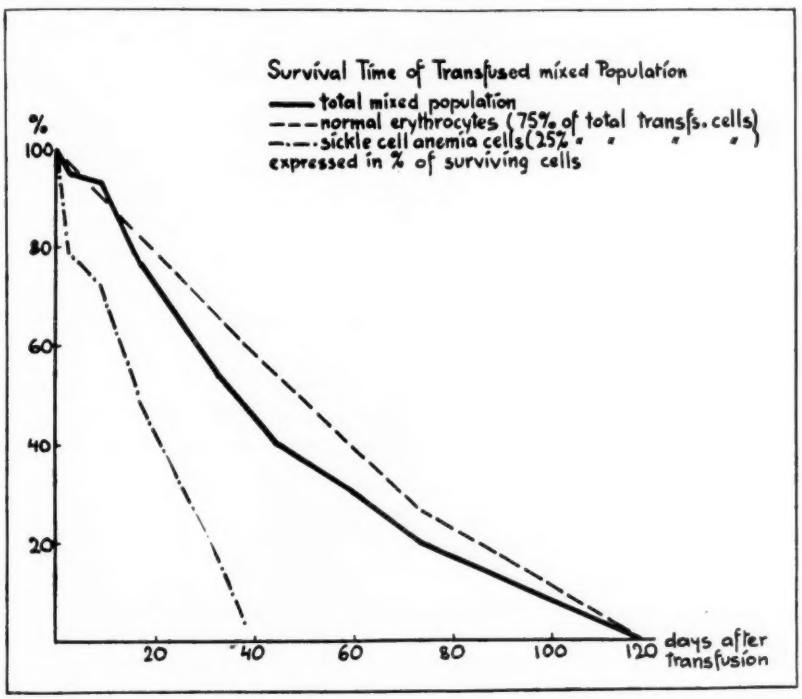

Fig. 3.—Survival time of transfused mixed erythrocyte population (normal and sickle cell anemia cells).*

Transfused normal erythrocytes: 740.000 = 100 per cent.
Transfused anemia cells: 286.000 = 100 per cent.

The phenomenon of the variable destructibility of different proportions of the red cell population in sickle cell anemia is essential for an understanding of certain dynamic aspects of the "aplastic" crisis. It explains why in the absence of any cell replacement the erythrocyte count will show a rather precipitous fall in some patients, and a less distinct decrease in others. It also permits an interpretation of the observation that a single blood transfusion apparently stopped the crisis in our case. Actually, after the destruction of the most "fragile" corpuscles the rate of hemolysis slowed down, and a few days later reticulocytes again were released into the circulation.

This analysis, therefore, indicates that an "aplastic" crisis in sickle cell anemia will develop only if the following two conditions come into play: (1) a rather complete cessation of erythrocyte production and release, and (2) the existence of red cells in the peripheral blood which must have the tendency to disintegrate within a very short period of time. The severity of the crisis will then be determined by the proportion of easily destructible cells present in the total erythrocyte population.

*See footnote, page 726.

DISCUSSION

The demonstration of a true "aplastic" crisis in sickle cell anemia is of clinical interest not only as an unusual complication but also because it reflects on the etiology and pathogenesis of this phenomenon in this disorder as well as in other chronic hemolytic syndromes. It is for this reason that some aspects of the general problems of the crisis will be discussed in greater detail.

(1) Etiology of the Erythrocytic Aplasia (Erythroblastopenia) of the Marrow.—The acute development of an aplasia of the erythropoietic tissue is the most important mechanism causing the "aplastic" crisis. So far this type of crisis has been described only in hereditary spherocytosis.[5] Since in this disorder splenic mechanisms are definitely involved, it has been considered that this marrow pattern may be induced by a pathologically hyperactive spleen.[6] However, the finding of this complication in sickle cell anemia demonstrates that the "aplastic" crisis is not specific for familial hemolytic jaundice. Furthermore, no evidence was present in our case to suggest any form of "hypersplenism."

On the other hand, clinical observations suggest that the severe depression of red cell production may be due to extrinsic, possibly infective, factors. It has been noted repeatedly that the hemolytic crisis in hereditary spherocytosis occurs either simultaneously or in rapid succession in several members of the same family, who may also show signs of an upper respiratory infection.[1, 6] Dedichen[2] found manifestations of such an infection in one person not afflicted with familial hemolytic jaundice, while a crisis occurred in hematologically abnormal members of the household. Horne and co-workers[4] placed a ferret in the home of such a family and the animal died shortly thereafter, apparently from "influenza." In our own observation, two siblings, both of whom had sickle cell anemia, were admitted to different hospitals on the same day. In one of these children the infection was severe, producing a typical virus pneumonia, and this patient developed the "aplastic" crisis. These clinical findings would seem to give strong support to the assumption that the erythrocytic aplasia represents a reaction of the bone marrow to an infective agent.

If this interpretation is correct, one would expect to see the appearance of such an abnormal marrow pattern in hematologically normal individuals during the course of similar infectious processes. It should be realized, however, that in such patients no "crisis" would occur, since, in the absence of any previously existing accelerated erythrocyte destruction, the rate of disappearance of the red cells from the circulation amounts to less than 1 per cent per day. Even if the erythropoietic aplasia persisted for one week, a decrease in the hemoglobin and erythrocyte levels might not be detected with the commonly used laboratory methods.

Systematic studies of the nucleated red cells of the marrow in virus pneumonia and other acute infectious diseases have not yet been undertaken. Recently, however, Gasser[8] described a transitory "erythroblastopenia" of the marrow in a nonanemic patient with atypical pneumonia. This author also found an erythrocytic aplasia in various other conditions, such as diphtheria, allergic reactions to drugs (Luminal, oleum chenopodii), after an insect bite,

and in patients with anaphylactoid purpura. The "erythroblastopenia" usually lasted about one week and was accompanied by reticulocytopenia and sometimes by moderate leuco- and thrombocytopenia in the peripheral blood. These observations suggest that the aplasia of the erythropoietic tissue may not only be caused by infective agents but also may develop on a toxic or allergic basis.

Histologically, the marrow pattern of an erythrocytic aplasia is characterized by a conspicuous lack of all but the earliest nucleated red cell precursors. Some observers have noted the presence of very large basophilic cells with a diameter up to 60 μ which most closely resemble the pronormoblast in staining qualities and nuclear structure, and they have considered them to be pathologic giant forms of this latter cell type.[5, 6, 8] Dameshek and Bloom[6] described the state of the marrow during the crisis in hereditary spherocytosis as a maturation arrest at the proerythroblast level. However, it should be emphasized that in our case as well as in those of Owren[5] and Gasser[8] the number of the very immature red cell progenitors was not found to be increased. The granulocytic series may exhibit a preponderance of immature cells,[5] but these findings seem to be inconstant.[6] In our studies we have not encountered any giant pronormoblasts, but there was a definite increase of plasma cells in the marrow at the height of the crisis. Since plasma cells were also found in the peripheral blood, this feature probably represents another response to the causative agent, similar to the eosinophilia observed by Gasser[8] in his cases of erythrocytic aplasia of allergic origin.

The leucopenia and slight thrombocytopenia (Table I) seen in our patient were also noted by Gasser in his nonanemic patients and were probably due to the infectious process. It should be mentioned, however, that similar blood changes also occurred in patients with spherocytic jaundice during the crisis[5, 6] and have been interpreted as being caused by a hyperactive spleen.[6]

The syndrome of "acute erythroblastopenia" requires much further study. It may well be the counterpart in the red cell series of disturbances in the production of granulocytes and platelets (agranulocytosis and thrombocytopenic purpura) due to infection or allergy.[8] No statement can be made at present in regard to the frequency of this particular reaction, since, being of short duration, it will not readily manifest itself in individuals with a normal red cell population and must, therefore, be particularly searched for. In patients with a chronic hemolytic anemia, however, the development of a transitory erythrocytic aplasia assumes practical significance, as it may precipitate a crisis, regardless of the specific etiology of the "erythroblastopenia."

(2) Pathogenesis of the "Aplastic" Crisis in Sickle Cell Anemia.—The severity of the crisis which may follow the development of a complete erythrocytic aplasia will depend entirely on the rapidity with which the erythrocytes disappear from the circulation, regardless of the mechanisms responsible for the accelerated destruction of the red cells.

The study of the survival time of the erythrocytes in our patient with sickle cell anemia clearly indicates that the course of the crisis can be correlated with the variable degree of destructibility of the different portions of the red cell

population. This phenomenon is not an isolated occurrence and has been observed repeatedly in sickle cell anemia.[13, 14] It is important, therefore, to recognize the factors which may account for this variable destructibility of the red cells.

It is now becoming more and more evident that the erythrocytes in sickle cell anemia are basically defective cells, and that no demonstrable extrinsic agents are responsible for the accelerated hemolysis. Cross determinations of the survival time[17] demonstrate that normal erythrocytes have a normal life span in the circulation of patients with sickle cell anemia, whereas sickle cell anemia cells disappear rapidly when transfused into normal individuals.[14] In contradistinction to hereditary spherocytosis, splenic destruction does not come into play. In many cases of sickle cell anemia due to thromboses of the larger vessels, the spleen may show such extreme fibrosis and shrinkage that at necropsy it is identified only with difficulty.[18] However, the severity of the anemia in such cases remains unaltered by this "autosplenectomy."

Intravascular sickling is commonly observed in patients with sickle cell anemia but occurs infrequently in persons with sicklemia (sickle cell trait).[19] Consequently it seems that anemia and trait cells differ in their tendency to exhibit the sickling phenomenon in vivo. Because of this, it has been suggested that in sickle cell anemia the mechanism of red cell disintegration consists of obstruction of capillaries by the sickling cells, causing stasis, thromboses, and premature dissolution of the trapped erythrocytes.[20]

Although a disposition for vascular occlusion undeniably exists in this disorder, such a pathogenetic mechanism does not satisfactorily explain the rather rapid elimination of a large mass of red cells. In our patient, more than one-half of the circulating erythrocytes disappeared within three days during the crisis without any clinical evidence of the presence of thromboses in the vascular system. Moreover, this hypothesis does not explain the established fact that erythrocytes from donors with the sickle cell trait, when transfused into patients with sickle cell anemia, have a normal survival time.[13, 14] Since the trait cells are capable of complete sickling if their oxyhemoglobin content is sufficiently reduced, the existence of a "thrombotic" mechanism of erythrocyte destruction (intimately connected with the sickle cell phenomenon) should result in a shortened life span of the transfused sicklemia cells. Furthermore, even if intravascular sickling is considerably reduced for a longer period of time by having individuals with sickle cell anemia breathe high oxygen concentrations, the rate of red cell elimination continues to be accelerated.[21, 22] For these reasons it has been assumed that the essential pathogenetic principle of sickle cell anemia resides entirely within the red cells.[13, 14] The defective erythrocytes are considered more vulnerable than normal ones to the continuous stresses within the circulation and thus disintegrate more rapidly.

Quite recently Pauling and associates,[23] using electrophoretic methods, have convincingly demonstrated that sickling of the erythrocytes occurs only in the presence of an abnormal hemoglobin. These investigators found that the anemia cells contain only this pathologic hemoglobin, whereas the trait cells contain a mixture of both normal and sickle cell hemoglobin. The concentration

of the latter apparently never exceeds 40 to 50 per cent of the total hemoglobin within the sicklemia cell. Thus the different sickling tendencies of the anemia and the trait cells can be correlated with the quantity of the abnormal hemoglobin present within these two types of erythrocytes.

Although this important contribution helps to clarify the mechanism of the sickling phenomenon, it must be emphasized that it does not furnish a complete explanation of the pathogenesis of sickle cell anemia. Patients with this disorder frequently show a different degree of anemia although the hemoglobin concentration of their erythrocytes does not vary considerably. Furthermore, the average survival time of transfused sickle cell anemia cells obtained from different patients varies over a range of from fifteen to sixty days[13, 14] and even different portions of the red cell population of the same patient reveal a markedly different degree of destructibility.

Many studies have shown that normal red cells which contain one type of hemoglobin only are destroyed at a constant daily rate according to their aging processes and the mechanical influences to which they are exposed within the circulation.[10, 11, 13, 14] Thus, with such erythrocytes, after transfusion into a suitable recipient, a straight "line of decay" is obtained in a graph when the percentage of surviving cells is plotted against time.[11] All other conditions remaining equal, if one assumes that the pathologic hemoglobin in the sickle cell anemia cells is the only abnormality determining their shortened life span, one would expect with these cells a straight but steeper "line of decay." Since, however, the elimination graph of transfused anemia cells is almost always a curved line,[13, 14] it seems necessary to postulate the existence of at least one other factor which also contributes to their premature disintegration.

The nature of this second factor and its possible relationship to the pathologic hemoglobin remain conjectural. If, for the reason previously outlined, one adheres to the hypothesis that the pathogenesis of sickle cell anemia is entirely of intraerythrocytic origin, then one may locate this additional factor within the stroma, thus assuming another structural anomaly of the erythrocytes of anemia.[13] The extent of this latter anomaly would then vary from case to case, and even from cell to cell.[14] However, equally justifiable is the postulation of an extrinsic mechanism, which may damage the pathologic erythrocytes selectively, and to a variable degree. No positive evidence for either of these assumptions is available at present. Whether intrinsic or extrinsic, it is this second factor, however, which, apart from the abnormal hemoglobin, assumes an important role in the pathogenesis of sickle cell anemia and thus also in the development of the "aplastic" crisis in this disease.

(3) Aplastic and Hyperplastic Hemolytic Crises.—Physiologically, the erythrocyte and hemoglobin levels of the blood represent the balance between red cell production and release and erythrocyte disintegration. In any hemolytic syndrome, regardless of the etiology, the rate of hemolysis is increased and a compensatory erythropoietic hyperplasia of the marrow with reticulocytosis ensues. In many patients with a chronic hemolytic anemia a new equilibrium between production and destruction develops and the peripheral erythrocyte value may again be relatively constant. Such a constancy at an anemic level

was apparently present in our patient and thus permitted a correlation between the rates of hemolysis during and after the "aplastic" crisis by means of survival time studies.

With this concept any "crisis" occurring in a hemolytic disorder may be interpreted as being caused by an acute imbalance of the forces which regulate the erythrocyte level. Although some investigators seem to imply that there exists only one type of crisis, it is now becoming clear that such a phenomenon may be elicited by quite different mechanisms. Three main varieties may be distinguished: (1) A sudden cessation in the delivery of cells without any exaggeration of the already accelerated hemolysis; (2) an abrupt augmentation of erythrocyte destruction above the precrisis level without any primary interference with production and release; and (3) a combination of both diminished or absent delivery and markedly exaggerated hemolysis. It should be noted that this classification considers only the dynamic aspects and does not refer to the various pathogenetic factors involved in bringing about any particular type of crisis.

Our survival time studies have supplied experimental evidence that the crisis which occurred in our patient belonged to the first category of this classification. Actually it was the lack of replacement of red cells due to the erythroblastopenic marrow which was of primary importance. Recognition that the "aplastic" crisis really is a "delivery" crisis is significant, because it is conceivable that inhibition of the release of red cells without any immediate disturbance in the erythropoiesis may produce the same phenomenon. This possibility is also suggested by the experiments of Reinhard and co-workers[21] and Tinsley and associates[22] who suppressed the output of erythrocytes in patients with sickle cell anemia and hereditary spherocytosis by an uninterrupted administration of oxygen in high concentrations for several weeks. An almost complete reticulocytopenia ensued, and since hemolysis continued at an abnormal rate, a considerable diminution of the circulating erythrocytes became noticeable. In two of these patients, in whom marrow studies were performed at the time of maximal reticulocyte depression, no marked changes in the number of nucleated red cells were found.[24] Thus the marrow in a "delivery" crisis may not always be "aplastic." Dameshek and Bloom[6] emphasize that a pathologically hyperactive spleen may not only inhibit the output of marrow cells but even be responsible for the maturation arrest of the erythropoiesis in the crisis of hereditary spherocytosis. The observation of an "aplastic" crisis in a patient with sickle cell anemia during the course of atypical pneumonia is more in favor of Owren's concept of a direct effect of the infection on the marrow, particularly since an erythroblastopenic marrow has also been observed in a nonanemic patient with the same infectious process.[8] However, the possibility of a "splenic inhibition" crisis in some cases should be kept in mind.

An imminent "delivery crisis" in a patient with a known hemolytic process should be suspected immediately if the absence of polychromatophilic red cells is noted on the film. If sufficient attention is paid to this readily detectable sign, more instances of this type of crisis may be diagnosed properly in the future in the various kinds of chronic hemolytic anemias.

It is now of great interest that cases of familial hemolytic jaundice have been observed in which a dramatic fall in the red cell level occurred although the marrow showed very active erythropoiesis and the output of reticulocytes was markedly increased.[12, 25] According to the "imbalance theory," this phenomenon can be explained only as a sudden exaggeration of red cell disintegration. The periodic exacerbations in erythrocyte destruction seen in patients with sickle cell anemia also seem to belong to this second "hyperhemolytic" type of crisis.

The pathogenesis of the hyperhemolysis has not yet been extensively investigated and is only poorly understood. In hereditary spherocytosis, increased splenic activity[6, 12] or formation of extremely maldeveloped spherocytes may be considered as possible causative mechanisms. Recently, by means of new methods (Coombs antiglobulin test[26] and use of trypsinized red cells[27]), autoimmune bodies have been demonstrated in the plasma of patients with familial spherocytic anemia. It is conceivable that sudden massive discharge of these immune bodies may produce an additional injury to the spherocytes and thus, at least in some instances, elicit a hyperhemolytic crisis.[28] No adequate explanation is available for the periodic hemolytic exacerbations in the course of sickle cell anemia. We could not detect autoimmune bodies during these episodes.

The "hyperhemolytic" type of crisis can be distinguished readily from the "aplastic" variety by the erythrocytic hyperplasia of the marrow and the elevated reticulocyte count. Although technically difficult to perform because of the existing emergency situation, comparative pigment, immunologic, and survival time studies during and after the crisis are greatly needed to enhance our understanding of the factors responsible for the hyperhemolysis.

The coexistence of absent red cell delivery and exaggeration of erythrocyte destruction above the precrisis level obviously represents the most complicated and most dangerous type of hemolytic crisis. One of the cases in the series of Dameshek and Bloom[6] is sufficiently well documented with marrow studies and life span determinations of transfused erythrocytes to be placed with certainty in this category. We recently observed one patient with malignant lymphoma in whom the marrow was almost devoid of erythropoiesis, resulting in extreme reticulocytopenia. Simultaneously an immunologic hemolytic process (positive Coombs test) existed. This patient required blood transfusions almost daily and was only incompletely relieved by splenectomy. In this instance the "erythroblastopenia" of the marrow was not transitory but chronic in nature because of the malignancy. Although Dameshek and Bloom[6] and Doan[12] consider that the crisis in hereditary spherocytosis is usually produced by "hypersplenism," our confirmation of Owren's concept in a patient with sickle cell anemia supports the hypothesis that several types of crises may exist in one and the same hemolytic syndrome.

As can be seen from this discussion, much remains to be learned about the various hemolytic crises. Since the causative mechanisms cannot be readily duplicated in animal experiments, many related problems can be approached only by clinical investigations under unfavorable "emergency" conditions. The

incidence of sickle cell anemia is, however, far greater than that of hereditary spherocytosis in some parts of this country. With the demonstration of a true "aplastic crisis" in the course of an infection in the former disorder, one may expect that similar cases may be observed more often in the future and thus a study on larger clinical material may become feasible.* To draw attention to such cases and the related hemotologic problems is the main purpose of this paper.

SUMMARY

The observation of an "aplastic" crisis which occurred during the course of clinically demonstrable virus pneumonia in a patient with sickle cell anemia is reported. On admission the patient showed reticulocytopenia, leucopenia, and erythrocytic aplasia of the marrow. Within three days the red cell level dropped to about 50 per cent of its previous level. Six days later numerous reticulocytes and nucleated red cells reappeared in the blood and the marrow had reverted to an extreme hyperplasia of the erythropoietic tissue.

The "aplastic" crisis is supposedly due to the combination of absent red cell production together with an unaltered rapid elimination of the defective erythrocytes from the circulation. It was attempted to demonstrate this mechanism in our case by determination of the survival time of the patient's erythrocytes seven months after his recovery. About 50 per cent of the transfused cells disappeared within three days—a finding in close agreement with the observed elimination of about half of the red cell mass during the crisis. These results, therefore, support the above-mentioned concept of the pathogenesis of the "aplastic" crisis.

Analysis of the survival time graphs of the erythrocytes of this and other patients with sickle cell anemia demonstrates that different portions of the red cell population show a variable degree of destructibility. The severity of the "aplastic" crisis will depend on the percentage of the most easily destructible cells present in the total erythrocyte population.

The etiology of the erythropoietic aplasia in our patient appears to be related to the infective agent causing the pneumonia. This interpretation is supported by reported instances of familial crises in hereditary spherocytosis associated with infections and the finding of an erythroblastopenic marrow in a hematologically normal patient with virus pneumonia.

The factors responsible for the variable destructibility of the erythrocytes in sickle cell anemia are discussed. It is emphasized that the recent discovery of abnormal hemoglobin in this disease does not fully explain this phenomenon. It seems necessary to postulate an additional extrinsic or intraerythrocytic factor which also contributes to the premature disintegration of these defective erythrocytes.

*Since this paper went to the printer, we have observed another case of "aplastic" crisis occurring in a 3-year-old girl with sickle cell anemia. This patient entered the hospital with signs of an upper respiratory infection. Her hemoglobin was 3.5 Gm., with a red cell count of 1.25 million. Sickled cells were seen on the film but no reticulocytes were present. Marrow studies showed an almost complete absence of nucleated red cell precursors. The reticulocytopenia lasted for seven days; afterward the reticulocytes rose gradually to 12 per cent, with a red cell count of 3.0 million. Serial marrow examinations revealed persistence of the erythroblastopenia for four days, followed by rapid transition to an extreme erythrocytic hyperplasia (E:G ratio of 4:1). No giant proerythroblasts were encountered, but increase of plasma cells in the peripheral blood and marrow was noticed again.

Evidence is presented that a precipitous decrease of the red cell level in an existing hemolytic syndrome may be produced by quite different mechanisms. Three main varieties of crisis may be distinguished. (a) The "*aplastic*" type, caused by a sudden cessation in the delivery of cells without any exaggeration of the already accelerated hemolysis; (b) the *hyperhemolytic* type, induced by an abrupt augmentation of erythrocyte destruction above the precrisis level without any primary interference with production and release; and (c) the *aplastic-hyperhemolytic* type, a combination of absent delivery of cells associated with markedly increased hemolysis. Our case belonged in the first category. The "aplastic" crisis should, therefore, be considered as a general phenomenon occurring in various hemolytic disorders and as not limited to hereditary spherocytosis.

An imminent "aplastic" crisis in a patient with a known hemolytic syndrome should be immediately suspected if the absence of polychromatic erythrocytes (reticulocytes) is noted on the film. If sufficient attention is paid to this readily detectable sign, more instances of this type of crisis may be diagnosed properly in the future.

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INFLUENCE OF SODIUM SALICYLATE AND SODIUM GENTISATE ON PROTEINURIA OF LIPEMIC NEPHROSIS

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MEMBERS of the salicylate group have been found to have an effect on various antigen-antibody reactions. The work of Swift[1] suggests an effect on the antibody forming mechanisms in rheumatic fever. Homburger[2] reports an impairment in the formation of anti-Rh agglutinins in rabbits and guinea pigs. Campbell[3] recently found salicylates to interfere with the antigen-antibody reactions of anaphylactic shock.

The intravenous injection of nephrotoxic sera obtained from rabbits produces a chronic renal disease in rats[4] that corresponds in many details to lipemic nephrosis with and without glomerular lesions as observed in children.[5] There is little doubt that the renal disease in rats is due to an antigen-antibody reaction. It thus was thought of interest to study the effect of salicylates on this disease. Meyer and Ragan[6] have reported that the anti-rheumatic action of salicylates is due to the oxidation product, gentisic acid. Sodium gentisate hence was included in this study.

METHODS

Rats of the Long-Evans and Whelan strains were used. They were fed Friskies* and had free access to water. Nephrotoxic nephrosis was induced when they were 5 to 6 weeks old. The technique described previously[4, 7] deviates in some details from the procedure used by Smadel, Swift, and Farr.[8] The urine was tested two to three times weekly for albumin by boiling and adding 3 per cent acetic acid until the reaction was slightly acid. Centrifuged sediments were examined in all specimens with more than a 1 plus reaction, which according to our technique corresponds to an excretion of 0.01 to 0.03 Gm. of albumin per twenty-four hours. Blood pressure readings were obtained with the foot method of Kersten, Brosene, Ablondi, and SubbaRow.[9] A 25 per cent solution of sodium salicylate was used. The daily dose was given by stomach tube in two divided doses. In control rats the lethal dose of salicylate, leading to death within six to nine days, was 170 to 200 mg. per day for 100 grams of body weight. Hyperpnoea, the characteristic symptom of salicylism in man, was not noted in rats. During the course of salicylate administration, 1.2 mg. of vitamin K (Hykinone) were given by subcutaneous injection twice weekly. A 15 per cent solution of sodium gentisate† was used. It was freshly prepared every third day. The lethal dose for control rats, leading to death within two to four days, was 300 mg. per 100 grams of body weight per day. Roseman, Pearson, and Dorfman[10] have found that the inhibition of hyaluronidase, which might play a role in the mechanism of action of salicylic and gentisic acid, is not due to pure gentisic acid, but to an impurity which probably is an oxidation product. In adaptation of their technique, a 10 per cent suspension of gentisic acid was neutralized with 1N NaOH, which resulted in an approximately 6 per cent solution of sodium gentisate. Before use, this solution was incubated in the presence of air for one hour at 37° C. and was freshly prepared every day. Both of these solutions were used. The daily dose was divided and given by stomach tube.

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*Dog food cubes, Albers Milling Co., Minneapolis, Minn.

†R. W. Greef & Co., Inc., New York, N. Y.

TABLE I. DETERMINATIONS OF SALICYLATES IN RAT PLASMA

RAT	WEIGHT (GM.)	DOSE PER DAY (MG. PER 100 GM. BODY WEIGHT)	DAYS OF TREATMENT	BLED AFTER LAST TUBING (HR.)	SALICYLATES (γ PER C.C.)
Control	260	40	2	1	581
Control	220	40	3	1	614
Control	330	50	2	16	683
Control	220	50	3	16	261
Nephrotic 387	310	40	10	3	515

RESULTS

Sodium Salicylate in Nephrotic Rats.—The concentration of salicylates was determined in the blood plasma of five rats (Table I). The amounts were almost twice as high as those observed in the plasma of patients treated with high salicylate doses for rheumatic fever. Fifteen rats with a well-established disease were treated (Fig. 1). In six of these, massive albuminuria had persisted uninterruptedly for more than two months. The remaining nine had shown a 4 plus albuminuria for one to seven weeks. Treatment was given for one to nine weeks. The daily dose varied between 25 and 100 mg. sodium salicylate per 100 grams of body weight. When loss of weight, loss of appetite, and listlessness

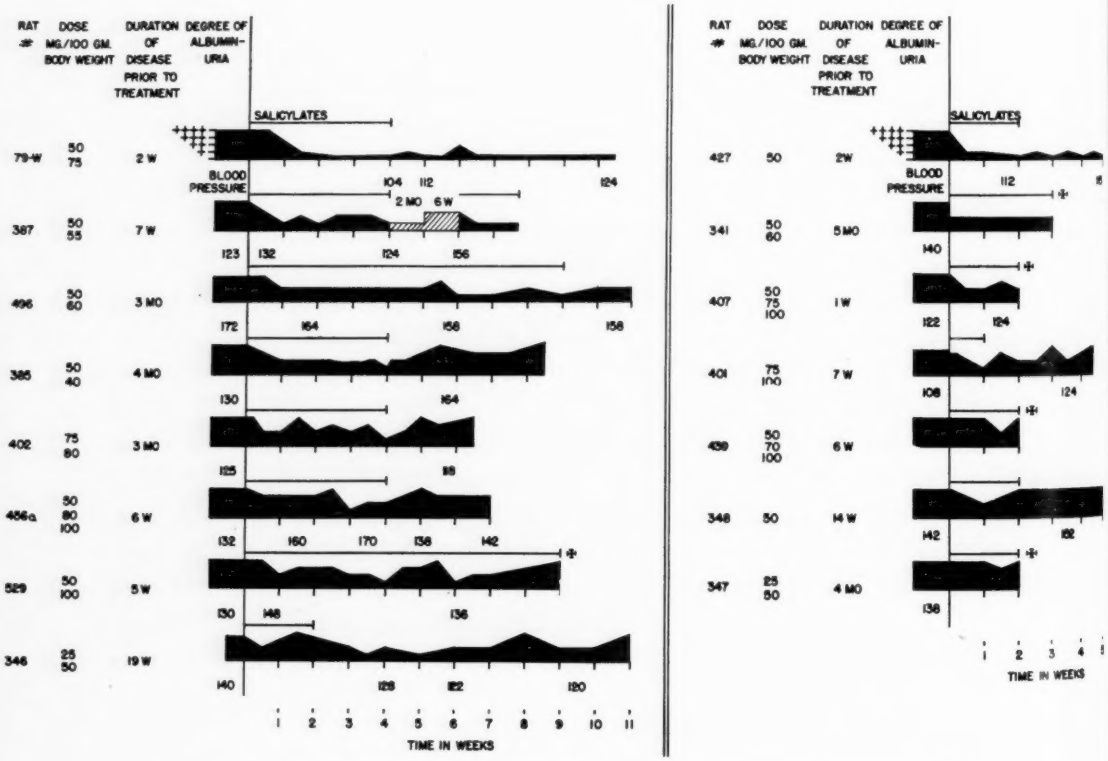

Fig. 1.—Effect of sodium salicylate on the proteinuria of fifteen nephrotic rats.

were noted, the gradual increase of salicylate was stopped. Before discussing the effect of this medication on the proteinuria of nephrotic rats, it has to be emphasized that a 1 plus albumin reaction is frequently, and a 2 plus reaction rarely, noted in apparently healthy control animals. Fig. 1 shows that the proteinuria was suppressed while salicylates were given in five (Rats 79-W, 427, 387, 496, and 401) and was unquestionably reduced in six (Rats 385, 341, 407, 402, 456a, and 529) of the fifteen treated animals. The first definite improvement was usually, but not always, noted within the first four to six days of treatment. In four other animals (Rats 439, 346, 348, and 347) the administration of salicylate had no effect on the proteinuria. None of these last four was given more than 50 mg. sodium salicylate per day. None of these four rats was treated for more than two weeks and in three of them the renal disease had persisted for more than three and one-half months before treatment was started. In all but two of the surviving animals the proteinuria increased to pretreatment levels one to eight weeks after the administration of salicylates had been discontinued. The two rats that did not relapse were, however, observed for only three and nine weeks respectively after treatment was discontinued. No definite influence on the blood pressure of these animals was observed.

Sodium Gentisate in Nephrotic Rats.—Nine nephrotic rats were treated with a daily dose of 100 to 250 mg. of sodium gentisate per 100 grams of body weight. They had had well-established renal disease that had been observed for three weeks to seven months prior to treatment. The medication was administered for three to four weeks. The commercial product was used in six, and sodium gentisate freshly prepared every day by adding NaOH to gentisic acid* was given to five animals. No influence of this medication on the proteinuria was noted in any of these rats.

Sodium Salicylate in Nephrotic Children.—Sodium salicylate† was used in three nephrotic patients. It was given by mouth in four divided doses every six hours. The daily dose was gradually increased according to tolerance and under control of the blood salicylate concentration. Vitamin K, 3 to 4 mg. a day, was given orally as long as salicylates were administered.

Patient M. G. (Fig. 2) was 7½ years old on admission on March 29, 1949. The disease began in February, 1947, when it was noted that her eyes, face, legs, and abdomen were swollen. No infection preceded the onset. The urine showed massive albuminuria, no hematuria, casts, and a specific gravity of 1020 to 1025. The serum proteins fluctuated between 3.5 and 4 Gm. per cent, the cholesterol between 568 and 750 mg. per cent, the nonprotein nitrogen was 34 to 42 mg. per cent, and the blood creatinine 0.5 to 1.1 mg. per cent. The blood pressure varied between 110/70 and 120/90. Because of intercurrent infections, edema and ascites, the patient had been admitted to the hospital four times within two years. On admission on March 29, 1949, she was free of edema and ascites and was a well-nourished and well-developed girl, with a blood pressure of 120/85. Because of the slight hypertension, the diagnosis of lipemic nephrosis, probably with some glomerular lesions, was made. It can be seen (Fig. 2) that the proteinuria decreased while salicylates were given, from a pretreatment level of 4 to 6 Gm. albumin per twenty-four hours, to 2 to 3 grams. Within twenty-four hours after the salicylate medication was discontinued, the values fluctuated between 5

*R. W. Greef & Co., Inc.
†Enteric coated, Lilly.

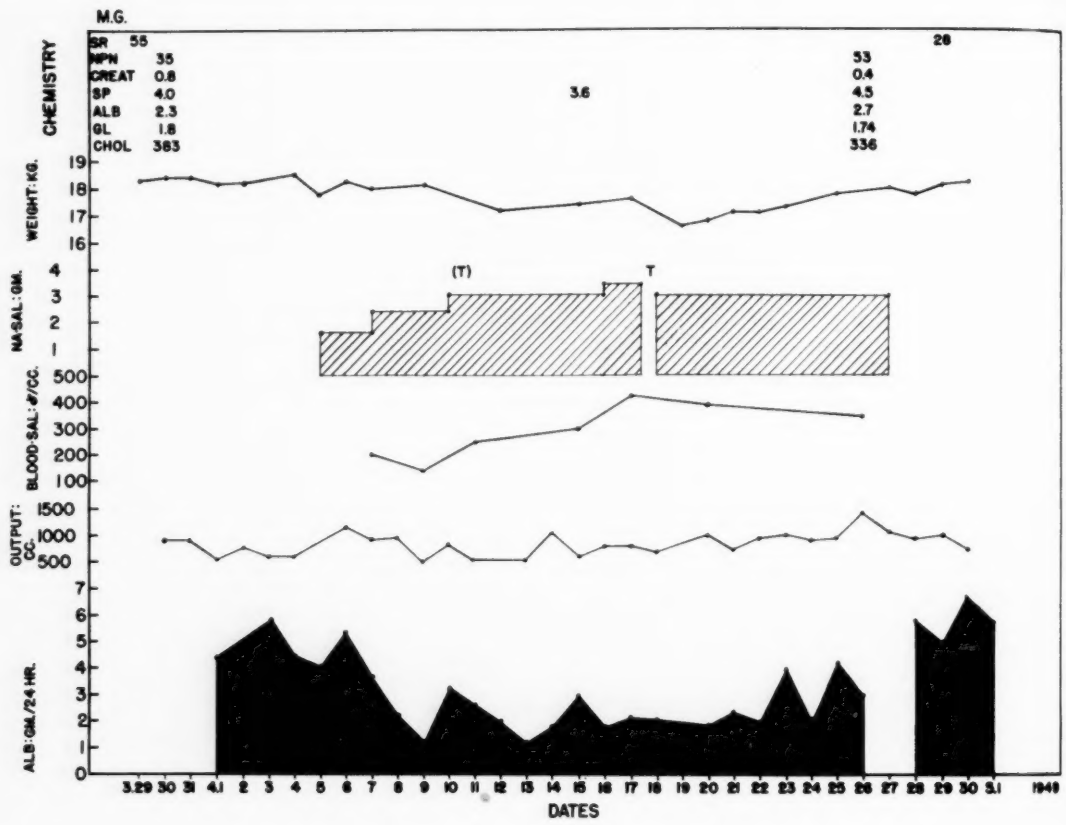

Fig. 2.—Effect of sodium salicylate on the proteinuria of Patient M. G.

and 7 grams. Inasmuch as this patient had no ascites and edema, it is believed that the loss in weight during that period was due to diminished food intake. Some lassitude, fatigue, and loss of appetite were noted while the blood salicylate concentration was between 300 and 400 γ per milliliter. No diuresis was observed. The changes in the blood chemistry before and after treatment are not of a magnitude that would permit a definite conclusion.

The second patient, R. G. (Fig. 3), was 4 years and 9 months old when admitted on June 13, 1949. He was first seen in February, 1947, three weeks after onset of the illness. At that time enlargement of the abdomen and edema were noted. The patient had had mild colds four months and one week respectively prior to his first admission. The urine examination showed a 4 plus albumin reaction, casts, a specific gravity of 1030 to 1040, no white or red blood cells. His blood pressure, when he was 2 years and 5 months of age, varied between 100/70 and 110/80. The nonprotein nitrogen fluctuated between 33 and 43 mg. per cent, the creatinine between 0.7 and 0.8 mg. per cent, the serum proteins 2.0 to 4.0 Gm. per cent, the cholesterol 492 to 1088 mg. per cent. In October, 1947, hematuria, with a 1 plus to 4 plus benzidine reaction, was noted for a period of two weeks. Because of the intercurrent infections and the need for abdominal paracentesis the patient had been admitted to the hospital ten times during the 2 years and 4 months of his illness. When hospitalized on June 13, 1949, the patient was well nourished and developed, had some edema and ascites, and the blood pressure varied between 110/65 and 125/80. The urine showed a 4 plus albumin reaction, a specific gravity of 1021 to 1035; 1 to 3 white but no red blood cells were seen in

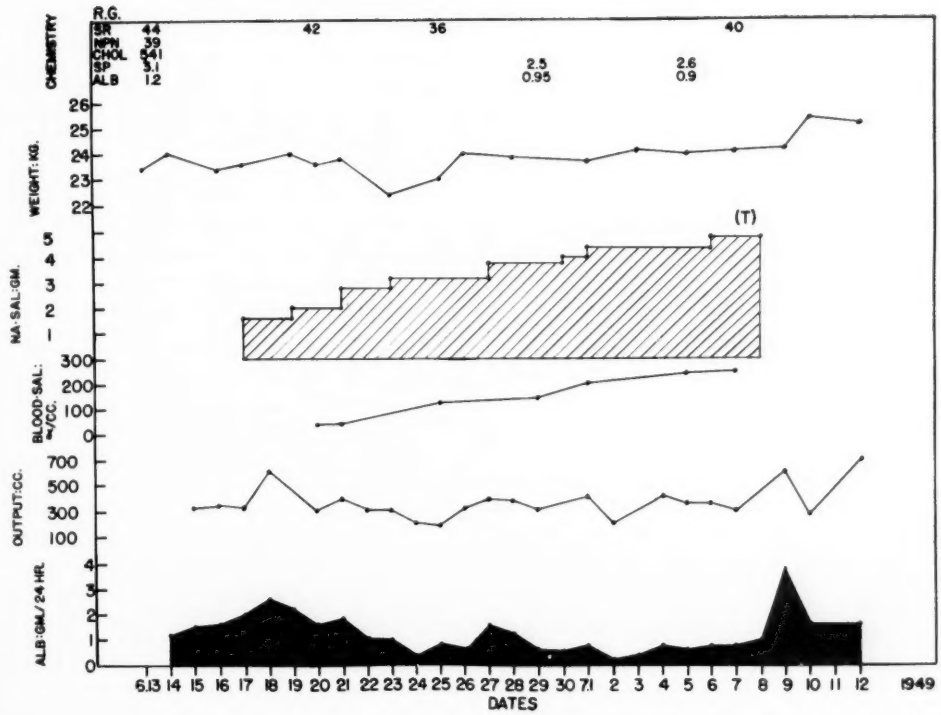

Fig. 3.—Effect of sodium salicylate on the proteinuria of Patient R. G.

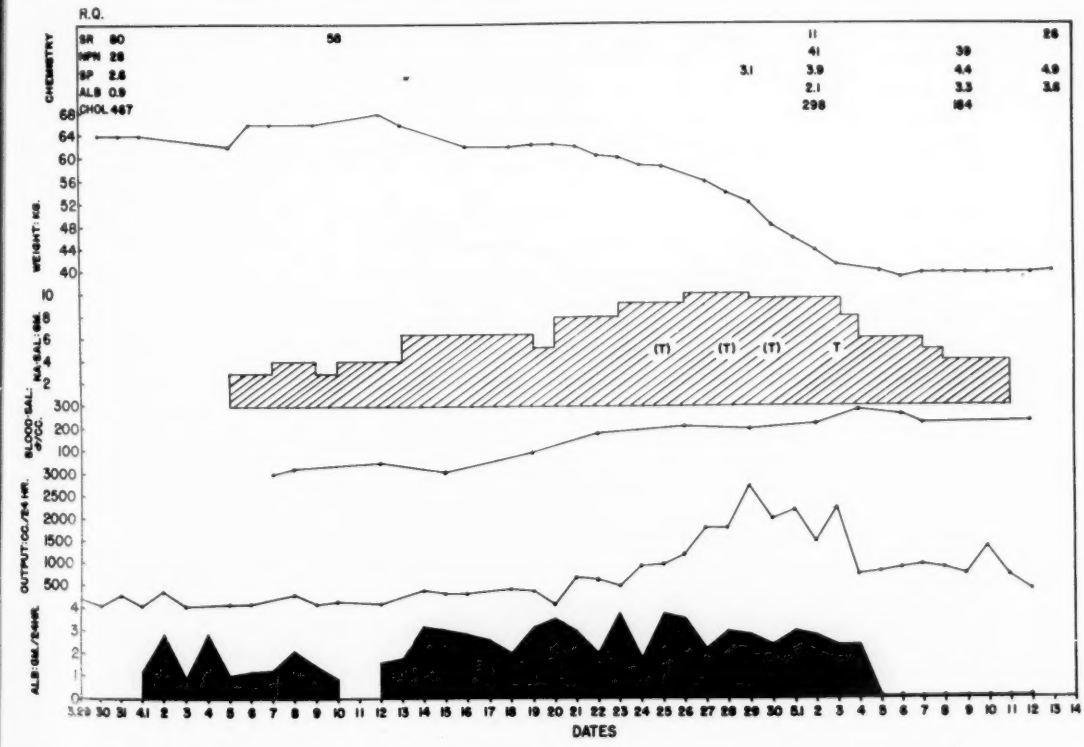

Fig. 4.—Effect of sodium salicylate in Patient R. Q. suffering from lipemic nephrosis.

the sediment and the benzidine reaction was negative. Because of an occasional elevated nonprotein nitrogen value, one period of hematuria, and a slight but almost continuous hypertension, the diagnosis of lipemic nephrosis with some glomerular lesions was entertained.

Sodium salicylate in increasing amounts was given for twenty-two days (Fig. 3). With a final dose of almost 5 Gm. daily, the blood salicylate concentration was only 250 γ per milliliter of plasma. The daily excretion of 1 to 2.5 Gm. of albumin in the urine which prevailed before treatment was 0.5 to 0.8 Gm. per twenty-four hours while salicylates were given. Within twenty-four hours after the salicylate medication was discontinued, the daily loss of urinary proteins was again bteween 1 and 3 grams. No loss in weight and no diuresis was noted and the blood chemistry did not improve during that period.

The third patient given salicylates was a 14-year-old girl, R. Q. (Fig. 4). The onset of the illness dates back to August, 1943, when she was 8½ years old and when it was first noted that she began to "swell." No infection preceded this onset. During the 5½ years of her illness, the patient was admitted eight times to the hospital because of ascites and edema and intercurrent infections of various nature. The urine always showed a 4 plus albumin reaction, a specific gravity of 1022 to 1030, and an occasional white cell but no red blood cells. The benzidine reaction was always negative. The nonprotein nitrogen varied between 24 and 32 mg. per cent, the creatinine between 0.8 and 1.9 mg. per cent; the serum proteins fluctuated between 1.5 and 5.0 Gm. per cent, the cholesterol between 202 and 600 mg. per cent. The blood pressure always was between 110/60 and 120/80. From March until September, 1946, the patient was free of edema, ascites, and albuminuria. This remission was noted after 200 ml. of plasma had been given intravenously every day for two weeks. In September, 1946, the patient showed edema of the face and ankles and had ascites soon thereafter; massive albuminuria was continuously noted from then until her admission on March 28, 1949. The diagnosis of lipemic nephrosis without apparent glomerular disease was entertained. Sodium salicylate was given in increasing amounts for thirty-seven days (Fig. 4). With a daily dose of 10 Gm. the concentration of salicylates in the plasma was only 200 γ per cubic centimeter. Increasing diuresis with progressive loss of edema and ascites started after fourteen days of treatment. Coincidental with this the patient showed signs of slight salicylate toxicity: apathy, listlessness, loss of appetite, and tinnitus. As the edema and ascites decreased, the blood salicylate concentration increased, in spite of a continuous reduction of the daily salicylate doses. The total amount of proteins excreted with the urine in twenty-four hours did not decrease appreciably. The amounts of protein per 100 c.c. of urine decreased, however, markedly. Quite suddenly, from the thirtieth to the thirty-first day of treatment, the amounts of protein excreted in the urine became too small to be determined quantitatively and the qualitative test for albumin became negative. Thirty days after salicylate administration was begun, the patient was free of edema and ascites and had lost 28 kg. (40 per cent of initial body weight) in weight. The serum proteins rose slowly and progressively to almost normal levels and the hypercholesterolemia decreased, perhaps somewhat more rapidly, to normal values. The initially rapid sedimentation rate of the red blood cells came down to normal values twenty-eight days after salicylate medication had been started. The patient has been examined every two to three weeks for over nine months since discharge from the hospital. The urine has remained free of albumin; the blood pressure, blood chemistry, and sedimentation rate have been normal at all times. She is on a free diet, has received no medication, and has been going to school uninterruptedly.*

DISCUSSION

The administration of large doses of salicylates has a definite effect on the proteinuria of nephrotoxic nephrosis in rats. In fifteen experiments, the albuminuria decreased to normal in five and was definitely reduced in six animals.

*While this manuscript was in preparation for publication, proteinuria recurred in this patient. She was readmitted with ascites and edema, hypoproteinemia and hyperlipemia, but without azotemia, hypertension, or hematuria. Administration of salicylates again was followed within two weeks by diuresis, loss of urinary proteins, and return to normal of all blood chemistry values.

Four failed to respond. These four were treated for only two weeks. A concentration of 500 to 600 γ of salicylate per 1 c.c. of blood plasma seems to be needed in rats. The first improvement of the proteinuria was usually noted within the first three to four days of treatment. It can, however, take two to four weeks to observe the full effect of the medication. In all but two (Rats 79-W and 427, Fig. 1) the diminution or suppression of the proteinuria did not last for more than one to eight weeks after the administration of salicylates had been discontinued. It is of interest to note that Rat 387 relapsed spontaneously after a remission of two months' duration. The readministration of salicylates was again followed by a decrease of the proteins lost in the urine. A cure may possibly be assumed in only two of the fifteen animals. Rat 79-W was sacrificed seven weeks after salicylate administration had been discontinued. The blood chemistry revealed normal values: BUN 30 mg. per cent, creatinine 1.2 mg. per cent, serum proteins 5.8 mg. per cent, cholesterol 50 and total lipids 290 mg. per cent. The histologic examination of the kidneys revealed no pathologic lesions. Rat 427 died accidentally after having been free of proteinuria for nine weeks. Histologic and chemical studies could not be obtained.

The observations on three nephrotic children are very similar. In two of these patients, who were given salicylates for twenty-two and twenty-three days respectively, evidence was obtained that additional glomerular disease was present. In these two children the excretion of proteins in the urine was decreased to half of the pretreatment level just as long as sodium salicylate was given. No further beneficial effect was noted. The third patient was older, tolerated up to 10 Gm. of sodium salicylate by mouth daily, and was given salicylates for thirty-seven days. Inasmuch as the concentration of salicylate in plasma only rose to 200 γ per cubic centimeter before anasarca subsided and continued to rise to 275 γ per cubic centimeter in spite of a reduction of the salicylate intake while diuresis occurred, it may be assumed that larger doses are needed in the presence of edema and ascites to establish a certain concentration in plasma. This is supported by the observation that salicylates were found to be present in ascitic fluid in almost the same concentration as in the blood plasma. On April 15, 1949, the salicylate concentration in the blood plasma of this patient was 10.3 γ per cubic centimeter and in the ascitic fluid 8.9 γ per cubic centimeter. The need for a progressive decrease of the daily dose in accordance with the loss of edema and ascites has to be emphasized. The need for the administration of larger doses while anasarca is present is also evident from the experience with Patient R. G. (Fig. 3), who at the age of 4 years and 9 months needed almost 5 Gm. of sodium salicylate to establish a blood concentration of only 250 γ per cubic centimeter.

The effect of salicylates on Patient R. Q. (Fig. 4) was astonishing. Diuresis started on the twenty-first day and had eliminated 20 kg. of fluids by the thirtieth day of treatment. By this time the albuminuria had completely subsided and one week later the blood chemistry revealed almost normal figures. In spite of two mild upper respiratory infections, this patient has remained free of symptoms for over nine months.*

See footnote, page 742.

Six additional nephrotic children 1 to 3 years of age were given salicylates orally. Because of listlessness, loss of appetite, and vomiting, the administration was discontinued three to six days after onset, at a time when blood salicylate concentration was not higher than 100 to 150 γ per cubic centimeter. The increased susceptibility of infants and young children to the toxicity of salicylates would seem to eliminate that age group, which comprises 60 to 70 per cent[5] of nephrotic children, and would greatly reduce any possible therapeutic application of salicylates. As two of three older patients have not shown more than a temporary reduction of the proteinuria, the effect of salicylates even in nephrotic children older than 3 to 4 years of age is described as an interesting observation rather than as a recommended therapeutic procedure. It may be that if salicylates are tolerated in amounts that do establish a concentration of 250 to 350 γ per cubic centimeter for several weeks, an occasional older patient may be benefited.

The mode of action by which salicylates exert an effect on the proteinuria in lipemic nephrosis represents a challenging problem. From the results obtained in rats one may conclude that action is not due to production of the oxidation product, gentisic acid. Furthermore, in rats we are dealing with a disorder that has initially been elicited by an antibody-antigen reaction. After this reaction once has occurred, the disease in most instances perpetuates itself, without any further administration of injurious antibodies. Salicylates exert their effect on the proteinuria of nephrotic rats as late as five months (Rat 341, Fig. 1) after the disease has been induced. It thus seems unlikely that their action is on the antibody-antigen reaction itself. Work is in progress to clarify this problem.

SUMMARY

The effect of sodium salicylate on nephrotoxic nephrosis was studied in fifteen rats. Renal disease was induced by the intravenous injection of nephrotoxic sera obtained from rabbits. Twenty-five to one hundred milligrams of sodium salicylate per 100 grams of body weight were given daily by mouth for one to nine weeks. Proteinuria was suppressed in five, quantitatively reduced in six, and remained unaffected in four animals. Relapse occurred in all but two rats.

Sodium gentisate was given in doses of 100 to 250 mg. per 100 grams of body weight to nine nephrotic rats for three to four weeks. This agent was without effect in all animals.

Sodium salicylate was given orally to three nephrotic children. Two of these patients had evidence of glomerular lesions. They were treated with increasing doses of sodium salicylates for twenty-two and twenty-three days respectively and their plasma-salicylate concentration rose to 250 and 350 γ per cubic centimeter. The proteinuria decreased in both children to half of the pretreatment level. No further benefit was noted. The third child suffered from lipemic nephrosis without apparent glomerular involvement. Salicylates were given for thirty-seven days and a plasma level of 200 to 300 γ per cubic centimeter was maintained for nineteen days. Complete elimination of edema and ascites

occurred, the proteinuria stopped, the blood chemistry and sedimentation rate returned to normal, and this patient has thus far remained free of symptoms for over nine months.

Application in younger children was not possible because of toxic reactions to salicylates in the dosages required.

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THE ROLE OF THE SPLEEN IN RADIATION INJURY AND RECOVERY

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INTRODUCTION

IT WAS recently reported by Jacobson and associates[1, 2] that a single dose of 600 r whole body x-radiation regularly produced a severe anemia, leucopenia, and thrombocytopenia in CF-1 female mice.* On the other hand, no anemia and a leucopenia and thrombocytopenia of lesser severity and shorter duration occurred in mice given this dose if the spleens were mobilized through an abdominal incision and lead-protected during irradiation. It was also shown that whereas the $LD_{50}/28$ days† for CF_1 female mice (whole body, single dose x-radiation) was in the neighborhood of 550 r, the $LD_{50}/28$ days for this strain of comparable age, weight, and sex was *circa* 1,000 r when the spleen was surgically mobilized and lead-protected during irradiation. This communication compares the effect of 1025 r on the blood and blood forming tissue of mice which have had lead protection of the surgically mobilized spleen during irradiation with the effect of this dose on mice without lead protection of the surgically mobilized spleen. A preliminary report on this work appeared elsewhere.[3] Reference will be made also to the previous experiments[1] in which a dosage of 600 r was used.

MATERIAL AND METHODS

The mice used in this study were CF-1 females and were 10 to 12 weeks old when the experiment began. The mice were kept in the animal room in a constant temperature (74° F.) for four to six weeks before use and were maintained on a diet consisting of Derwood‡ and water ad libitum before and during the experiment.

Experimental Procedure.—This experiment consisted of a study of four groups of animals as indicated in Table I. Mice in Group I were untreated controls. The mice in Groups II, III, and IV were anesthetized with Nembutal (0.072 mg. per gram mouse in a 1:10 dilution) given intraperitoneally. The abdomen was shaved, an incision was made in the left upper quadrant, and the spleen was brought out through the incision. This procedure has been described in detail elsewhere.[1] Mice in Groups III and IV were irradiated with 1025 r whole body x-radiation except that during the irradiation the mobilized spleens of the mice in Group IV were placed in lead boxes one-quarter inch thick with an opening for the main pedicle. The mobilized spleens of the mice in Group III were placed in gauze moistened with warm saline during radiation. The mobilized spleens of the mice in Group II (operated controls) were placed in lead boxes for a period equal to the time that the spleens of mice in Group IV were thus contained. The radiation required approximately fifteen minutes, after which the spleens of mice in Groups II, III, and IV were returned to

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*CF-1 raised by Carworth Farms. Homozygous for the recessive genes aa bb cc.

†The dose required to produce death in one-half the animals in twenty-eight days.

‡Manufactured by the Derwood Mill, Derwood, Md. Consists of skim milk, ground wheat, yeast, corn oil, salt, iron citrate, shark liver oil, and Delsterol.

the abdominal cavity and the operative incisions sutured. The animals were weighed at the beginning of the experiment and at daily intervals for the first nine days, then every three or four days for twenty-eight days.

TABLE I. PREPARATION AND TREATMENT OF ANIMALS*

GROUP	NUMBER OF ANIMALS	PREPARATION OF ANIMALS	TREATMENT
Group I	40	None	None
Group II	50	Anesthetic (Nembutal) Surgical mobilization of spleen	None
Group III	45	Anesthetic (Nembutal) Surgical mobilization of spleen	1025 r whole body x-radiation including spleen
Group IV	30	Anesthetic (Nembutal) Surgical mobilization of spleen	1025 r whole body x-radiation with spleen lead-protected

*These animals used for hematologic studies only.

Dosimetry.—The x-rays administered in these experiments were generated in a 250 kv. machine operating at 15 milliamperes. A 0.25 mm. copper filter was used. The half-value layer in copper of the filtered beam using 240 kv. was 1.0 millimeter. The exposures were measured with a Victoreen condensor r-meter equipped with a 100 r chamber. Measurements were made in air at the position occupied by the center of the animal's body. The dose rate averaged 58.9 r per minute at 55 centimeters. Backscatter from the lead shield used for lead protection of the spleen added an average of 5 per cent to the dose measured as described. To make Group III and Group IV comparable, the lead shield was placed against the body of the animals of Group III during irradiation in the same manner as it was in Group IV animals.

Histologic Studies.—Histologic studies were made on all four groups of animals. The mice were sacrificed at intervals as shown in Tables II and III. The tissues taken for study included the thymus, liver, spleen, lymph nodes from root of mesentery, small intestine including a Peyer's patch just proximal to the cecum, bone marrow (femur), adrenal, and kidney. The tissues were fixed in Zenker-Formol, embedded in nitrocellulose, cut at 8 or 6 μ and stained with hematoxylin-eosin-azure II. In addition to the animals sacrificed as stated, animals which died at the various intervals after irradiation were autopsied and the tissues fixed and cut as described. The purpose of this procedure was to determine if failure of hematopoietic regeneration in the spleen-protected group could be correlated with failure of the animal to survive.

Hematologic Studies.—The studies of the blood taken from the tail vein of the mouse, using standard techniques, included the following:

Hemoglobin in grams per 100 cubic centimeters.
Erythrocytes per cubic millimeter.
Leucocytes per cubic millimeter.
Differential leucocytes (counting chamber method).
Plasma platelet counts.[4]
Hematocrits (Kato).
Reticulocytes (per cent of erythrocytes).

RESULTS

All the mice in Group III with the spleen unprotected died within the first thirteen days after irradiation, as was expected, since 1025 r whole body x-radiation is several hundred roentgens above the LD_{100} (Table IV). No deaths

TABLE II. CELLULARITY OF THE BONE MARROW EXPRESSED IN PER CENT OF NORMAL AT INTERVALS AFTER IRRADIATION; PER CENT EXPRESSED AT EACH INTERVAL REPRESENTS AVERAGE (OF ESTIMATION) OF ALL ANIMALS SACRIFICED AT THAT INTERVAL

GROUP*	TREATMENT	NUMBER OF ANIMALS SACRIFICED	LEAD PROTECTION OF SPLEEN	INTERVAL AFTER IRRADIATION (DAYS)														
				1	2	4	5	6	7	8	9	10	11	12	13	15	29	37
Group I	0	33	No	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Group II	Operation only	45	No	100	100	100	100	100	100	100	100	100	100	100	100	100	10	100
Group III	1025 r whole body	77	No	20	10	0	0	0	0	0	0	0	10	10	25	No survivors		
Group IV	1025 r whole body	45	Yes	20	10	15	20	30	75	100	100	100	100	100	100	100	100	100

*Normal cellularity of marrow expressed as 100 per cent.

TABLE III. CELLULARITY OF THE LYMPH NODES AND PATCHES OF PEYER EXPRESSED IN PER CENT OF NORMAL AT INTERVALS AFTER IRRADIATION; PER CENT EXPRESSED AT EACH INTERVAL REPRESENTS AVERAGE (OF ESTIMATION) OF ALL ANIMALS SACRIFICED AT THAT INTERVAL

GROUP*	TREATMENT	NUMBER OF ANIMALS SACRIFICED	LEAD PROTECTION OF SPLEEN	INTERVAL AFTER IRRADIATION (DAYS)														
				1	2	4	5	6	7	8	9	10	11	12	13	15	29	37
Group I	0	33	No	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Group II	Operation only	45	No	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Group III	1025 r whole body	77	No	15	0	0	0	0	0	0	0	0	0	0	0	No survivors		
Group IV	1025 r whole body	45	Yes	20	10	20	30	50	75	100	100	100	100	100	100	100	100	100

*Cellularity expressed in terms of number of cells in specimen compared with normal but does not take into account any reduction in the over-all size of the tissue.

TABLE IV. SURVIVAL OF MICE FOLLOWING SINGLE TOTAL BODY X-IRRADIATION WITH AND WITHOUT LEAD PROTECTION OF THE SPLEEN*

GROUP	DOSE	SPLEEN SHIELDED	TOTAL NUMBER OF MICE	NUMBER OF SURVIVORS	PER CENT OF SURVIVORS	TIME DISTRIBUTION OF DEATHS (DAYS)													
						3	4	5	6	7	8	9	10	11	12	13	14	15	16-30
I	0	No	11	11	100														
II	0	No	16	16	100														
III	1025	No	28	0	0	1	5	4	5	4	4	3	1	1					
IV	1025	Yes	68	40	41.2	1	1	12	7	5	6	1	5	1		1			

*These data agree with survival data previously published.

occurred in Group I and Group II (untreated controls and operated controls respectively) during the twenty-eight day period of observation. Forty-two per cent of the animals in Group IV (1025 r whole body x-radiation with lead protection of the surgically mobilized spleen) survived the twenty-eight day period of observation.

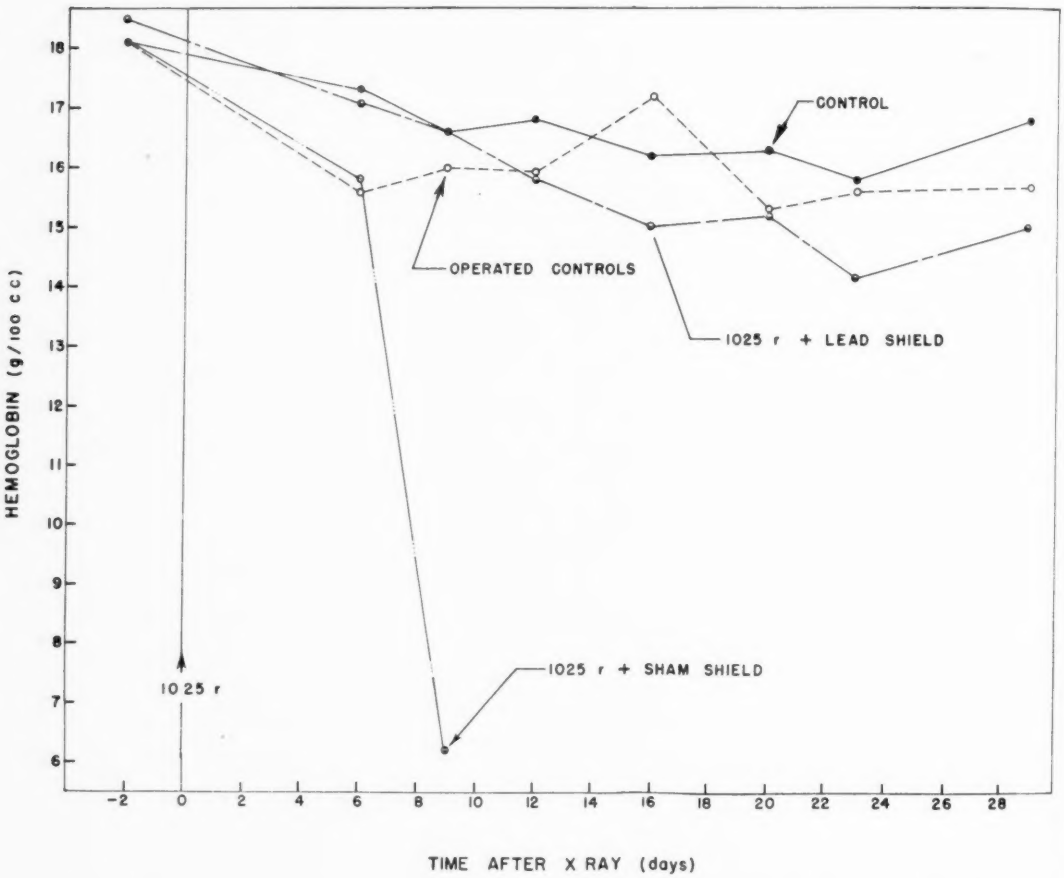

Fig. 1.—The hemoglobin values of control mice and mice exposed to 1025 r with and without lead protection of the spleen.

Hematologic Studies.—The mean hemoglobin, erythrocyte, and hematocrit values of mice in Group IV with lead-protected spleens were only slightly reduced when compared with the mean values of the control Groups I and II (Figs. 1 to 3). These values, however, were markedly and sharply reduced in the few animals that survived through the ninth day in Group III which received whole body radiation with spleens unprotected. The mean reticulocyte value of the mice in Group III with the spleen unprotected was reduced to less than 0.1 per cent by the sixth day and remained thus depressed through the ninth day. Death precluded further sampling of this group beyond the ninth day. The mean reticulocyte value of the mice in Group IV with lead-protected spleens was 3.1

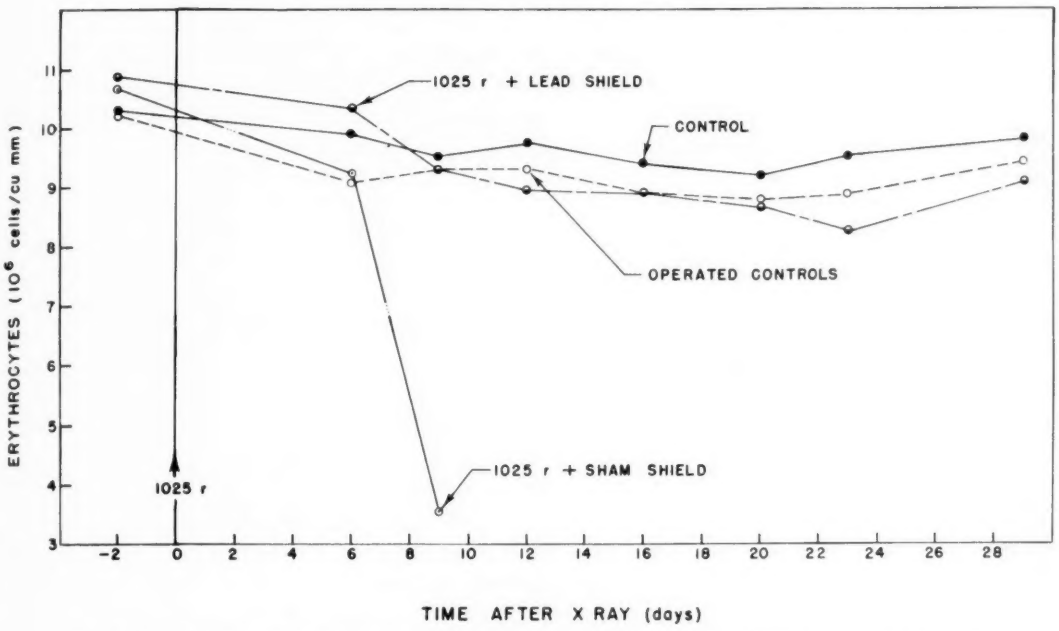

Fig. 2.—The erythrocyte values of control mice and mice exposed to 1025 r with and without lead protection of the spleen.

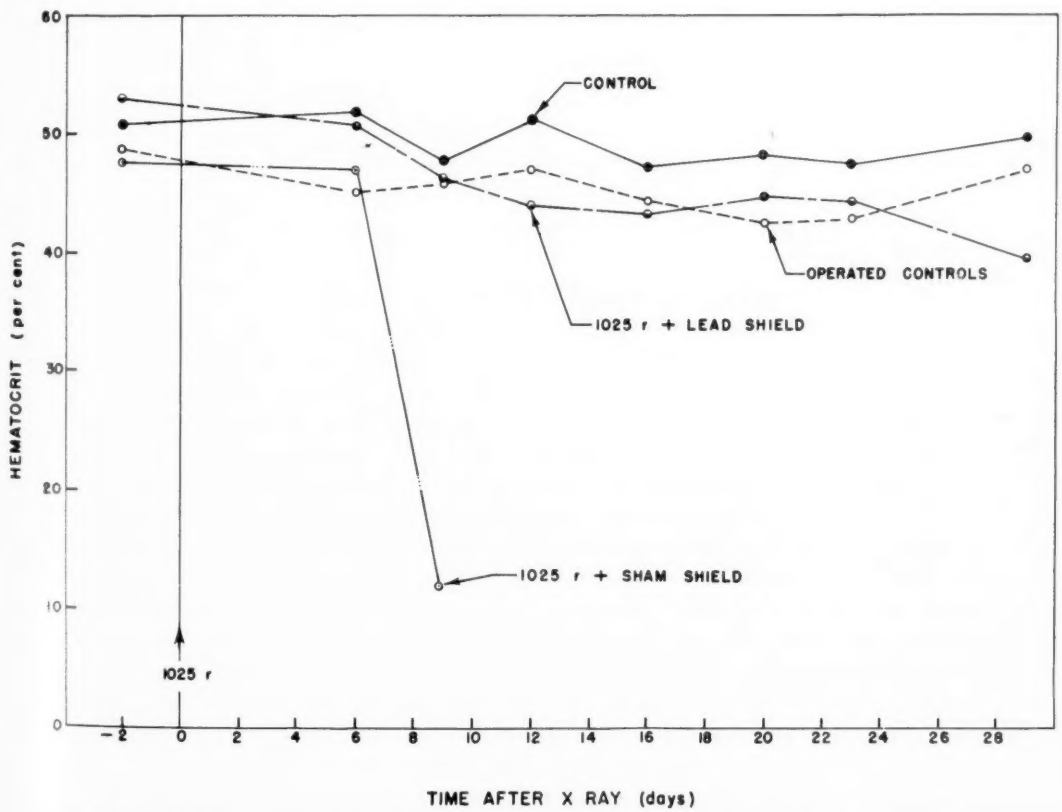

Fig. 3.—The hematocrit values of control mice and mice exposed to 1025 r with and without lead protection of the spleen.

per cent in six days, 5.9 per cent by the ninth day, 8.1 per cent by the twelfth day, and 9.1 per cent by the sixteenth day (Fig. 4). The mean platelet value of the animals in Group III with the spleen unprotected fell to 101,000 per cubic millimeter before death occurred, whereas the platelet value of the mice in Group

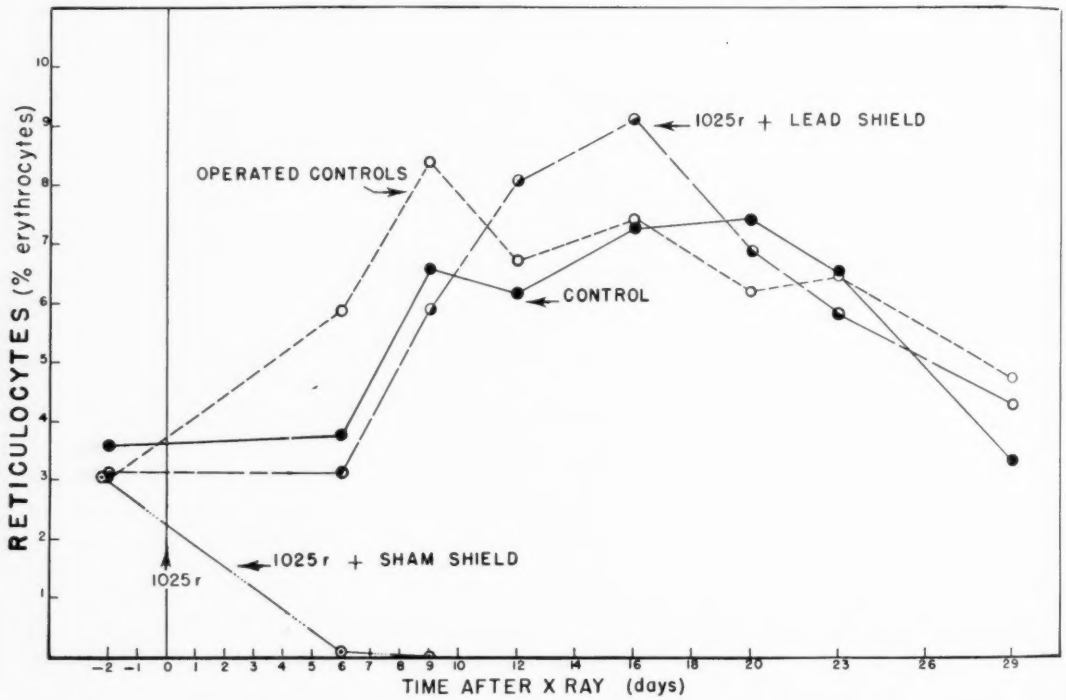

Fig. 4.—The reticulocyte values of control mice and mice exposed to 1025 r with and without lead protection of the spleen.

IV with the lead-protected spleens reached a minimum of 275,000 per cubic millimeter by the ninth day and rose to a normal value by the sixteenth day (Fig. 5). The mean leucocyte value of the mice in Group III with the unprotected spleens was reduced to 285 cells per cubic millimeter by the sixth day and remained below 700 per cubic millimeter in the few animals that survived through the ninth day after irradiation. The mean leucocyte value of the mice in Group IV that had lead-protected spleens fell maximally to 1,200 per cubic millimeter by the sixth day and rose to normal values by the ninth day after irradiation (Fig. 6). The mean heterophil and lymphocyte values of the Group III mice with the spleens unprotected fell maximally by the sixth day and remained low until death. In the mice of Group IV with the lead-protected spleens, the heterophil and lymphocyte values were reduced to 347 and 1,023 per cubic millimeter respectively by the sixth day (Figs. 7 and 8). By the ninth day the heterophil values had returned to normal and remained above normal during the period of observation (twenty-nine days), whereas the lymphocytes had risen to *circa* 4,500 per cubic millimeter by the ninth day and remained only slightly below normal values through twenty-nine days.

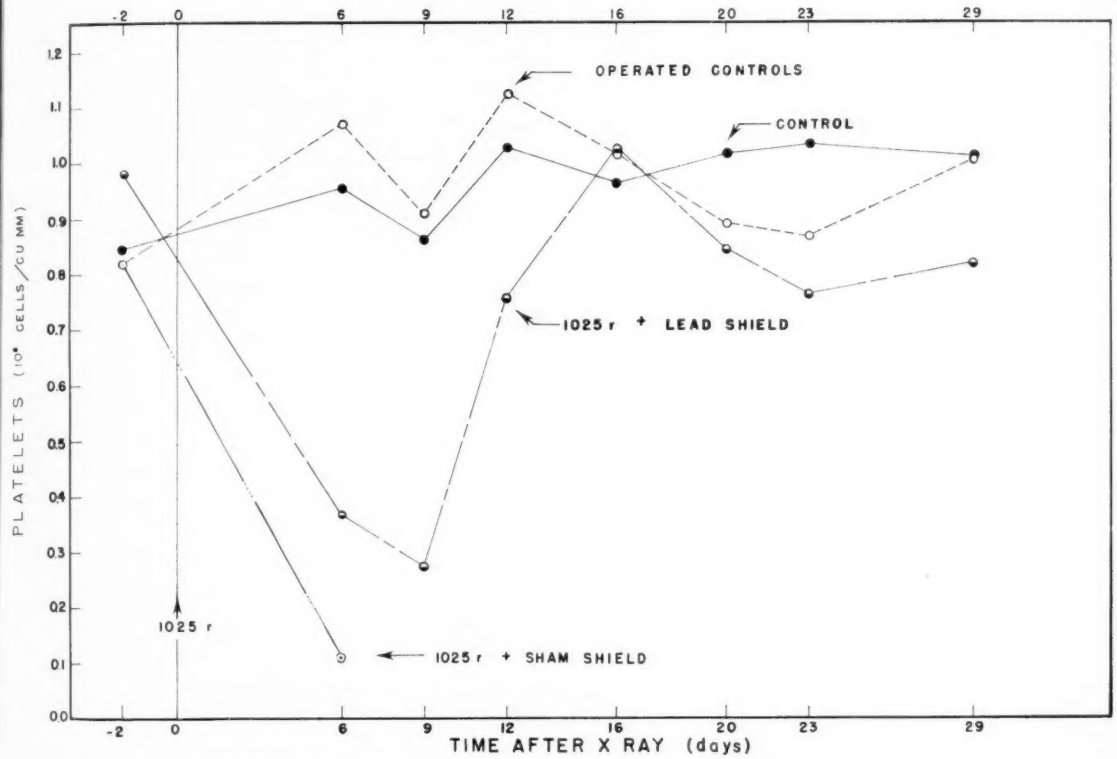

Fig. 5.—The platelet values of control mice and mice exposed to 1025 r with and without lead protection of the spleen.

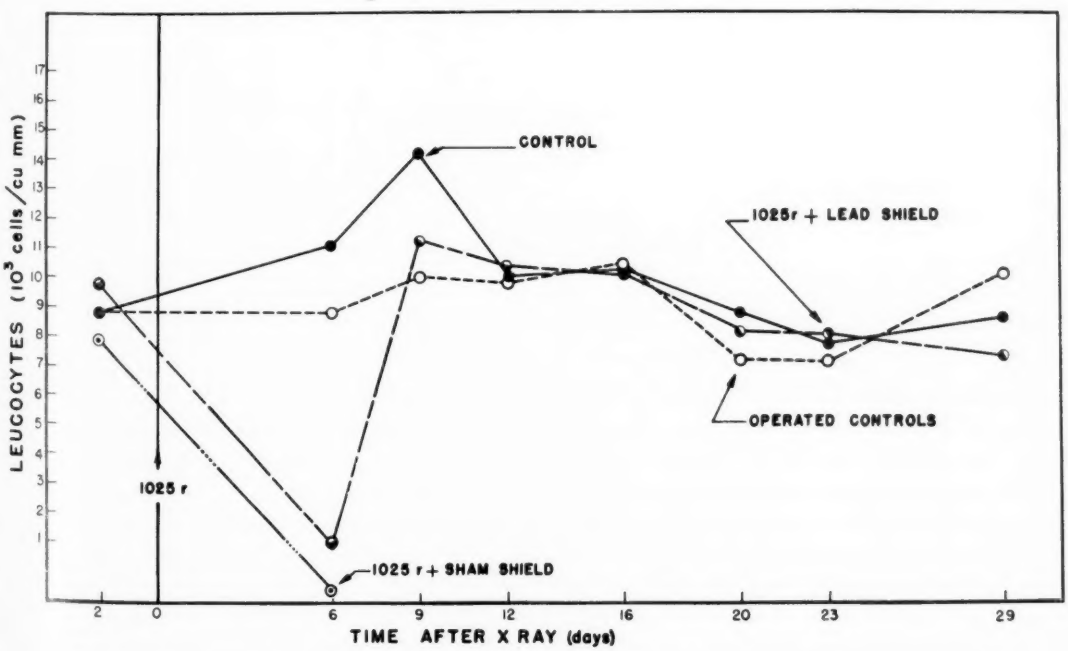

Fig. 6.—The leucocyte values of control mice and mice exposed to 1025 r with and without lead protection of the spleen.

Weight.—The mean weight of the animals in Group III that received whole body x-radiation inclusive of the spleen dropped gradually from *circa* 23 grams before irradiation to 13.5 grams on the tenth day. Death of all animals in this group prevented further weighing. The mean weight of the mice in Group IV

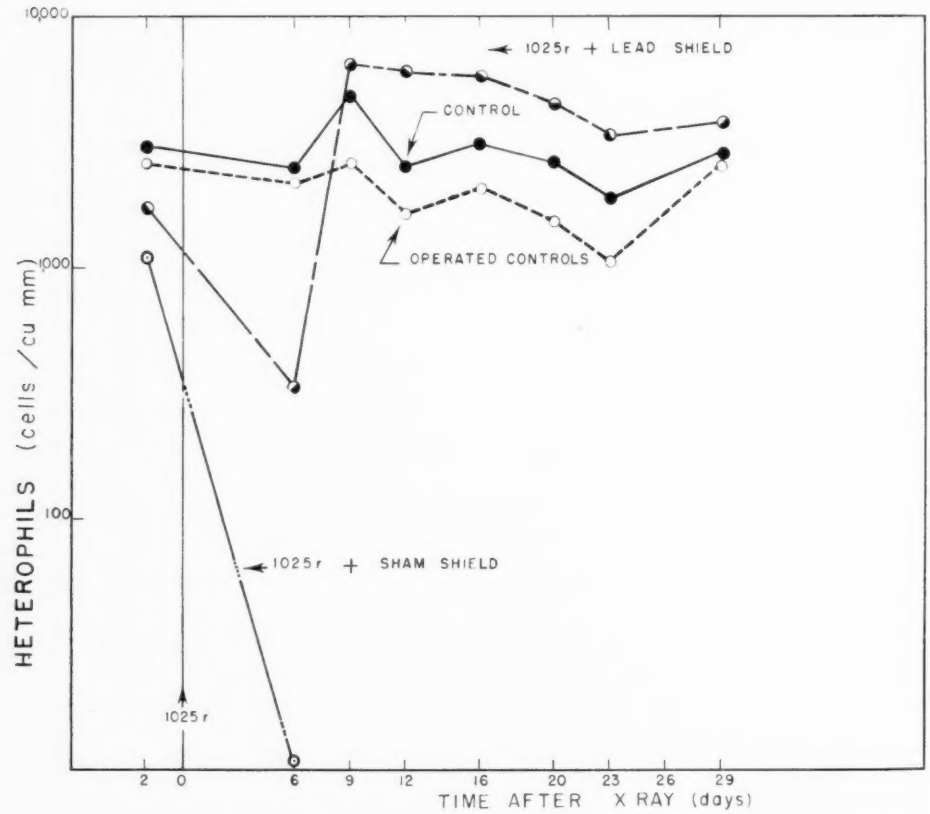

Fig. 7.—The heterophil values of control mice and mice exposed to 1025 r with and without lead protection of the spleen.

that had lead-protected spleens, however, dropped to a minimum of 16.5 grams by the sixth day and the mice returned to approximately their original weight by the eighteenth day (Fig. 9).

Histopathology.—Study of regeneration of hematopoiesis in mice after the destructive action of a single dose of 1025 r whole body x-radiation is necessarily incomplete because of the fact that death occurs so early. As noted in Table II, two mice survived to the thirteenth day in these experiments, but only by irradiating large numbers of animals was it possible to obtain this limited number of survivors for sacrifice at this interval. On the other hand, lead protection of the surgically mobilized spleen permits an indefinite survival of *circa* 42 per cent of mice exposed to this dose and this study of the regenerative phase of hematopoiesis is possible.

Bone Marrow: Marrow of control groups (I and II): The femoral bone marrow of normal untreated control mice (Group I) and mice which have had an operation only (Group II) at any of the intervals studied varies somewhat in cellularity. In general, hematopoietic tissue fills the marrow space except for

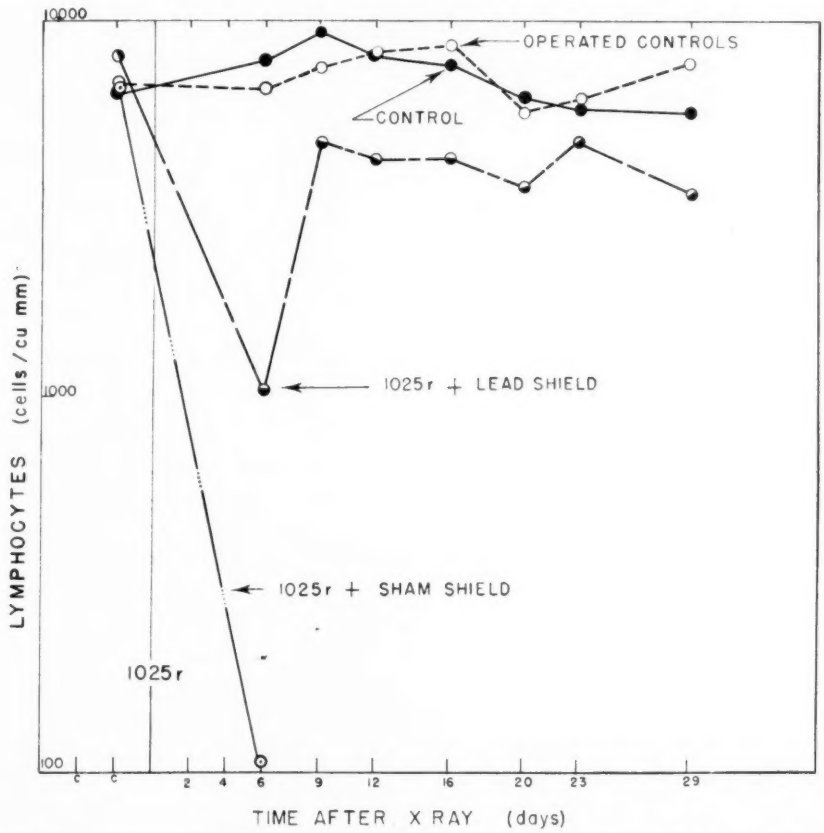

Fig. 8.—The lymphocyte values of the control mice and mice exposed to 1025 r with and without lead protection of the spleen.

blood sinuses which vary in prominence. In some animals the epiphysis, metaphysis, and diaphysis are so packed with cells that blood sinuses are very inconspicuous. In others the sinuses are prominent and the free hematopoietic cells less closely packed about them. On the average, erythroblasts constitute roughly 30 per cent of the marrow cells; the balance of the hematopoietic cells of the marrow are made up largely of heterophils and heterophil precursors and scattered megakaryocytes.

Marrow of group exposed to 1025 r without lead protection of the spleen (Group III): As is indicated in Table II and Fig. 10, the bone marrow of mice which had no lead protection of the spleen during irradiation (Group III) was essentially devoid of hematopoietic cells by the fourth day after irradiation. Except for a moderate prominence of plasma cells, the bone marrow at this interval and succeeding intervals studied through the tenth day was composed

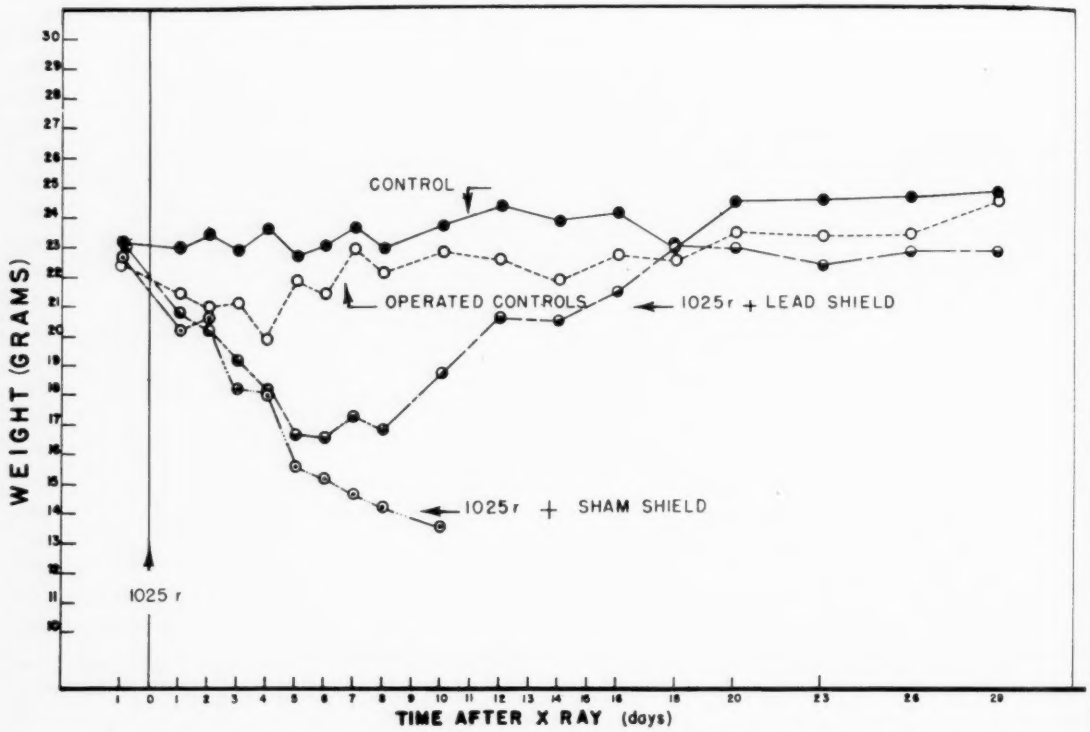

Fig. 9.—The weights of control mice and mice exposed to 1025 r with and without lead protection of the spleen.

largely of dilated blood sinuses, a variable amount of homogeneous bluish-staining gelatinous material, and a striking increase in spindle-shaped cells which, in most specimens, replaced the metaphyseal and epiphyseal portion of the marrow and in some specimens were prominent throughout the diaphysis as well (Fig. 11). The microscopic appearance of this tissue was comparable in many respects to the atypical fibrous metaphyseal marrow spaces observed in mice after injection of radiostrontium (Sr^{89}).[5] Beginning on the eleventh day, regeneration of the bone marrow was first apparent as a single focus or several small foci in the diaphysis. At the twelve-day interval such foci of regeneration were again apparent and on the thirteenth day the general cellularity of the diaphysis was approximately 25 per cent of normal. The atypical "fibrous" marrow in the metaphyseal and epiphyseal marrow persisted through the thirteenth day. The feeble attempts at regeneration in the marrow apparent at the intervals just described appeared to consist largely of erythroblastic precursors and a few scattered immature and mature megakaryocytes and granulocytes. Mitoses in the cells of these foci were prominent, whereas in the specimens inclusive of the fourth through the ninth days mitoses were absent.

Bone marrow of mice given 1025 r with lead protection of the spleen (Group IV): At the early intervals (one and two days) after irradiation, the bone marrow of Group III (spleen unprotected) and Group IV (spleen protected) was very similar in appearance, showing progressively fewer residual

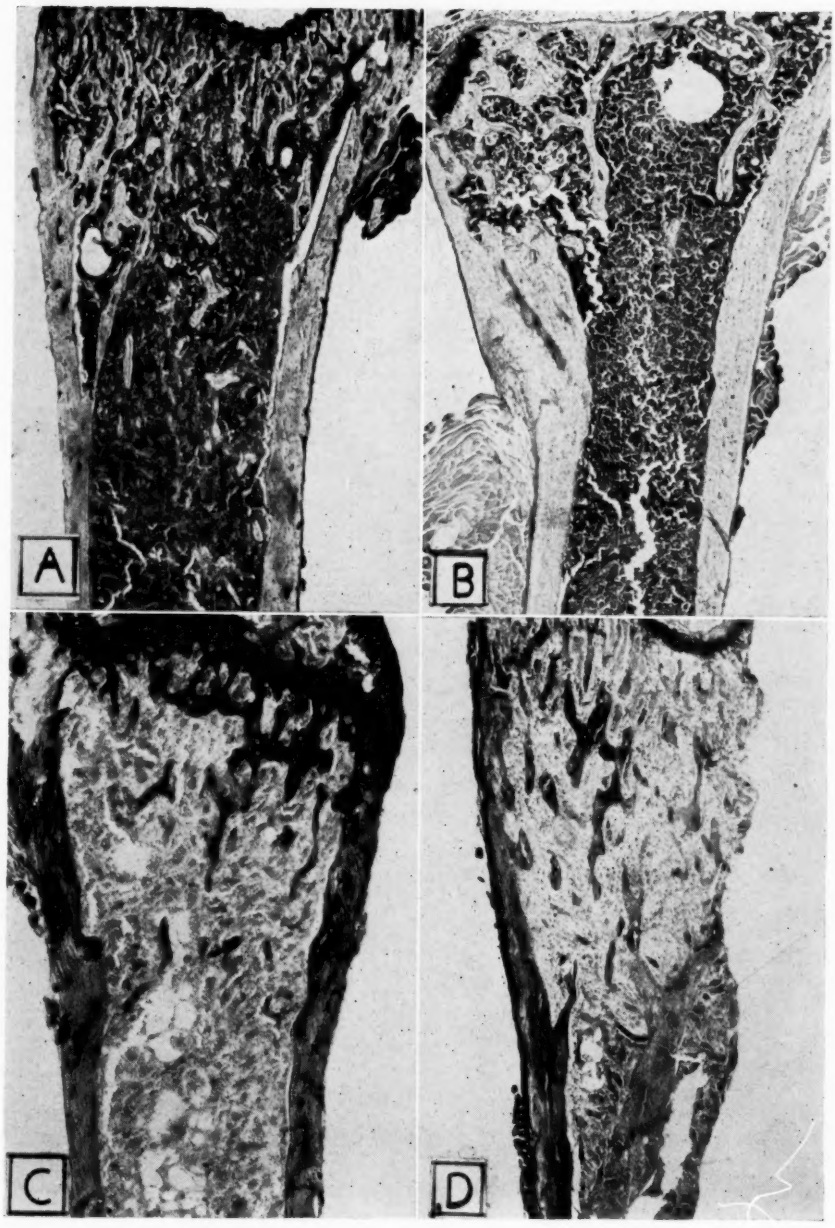

Fig. 10.—Femoral marrow of mice four days after exposure to 1025 r whole body X-radiation. *A*, Control untreated (Group I); *B*, control operated only (Group II); *C*, whole body X-radiation without spleen protection (Group III); *D*, whole body X-radiation with spleen protection (Group IV).

Note general acellularity except for hemorrhage, dilated vascular spaces, and atypical fibrous tissue in metaphysis of *C*, and atypical fibrous tissue in the metaphysis and clear-cut population of the diaphysis with hematopoietic cells in D. (Hematoxylin-eosin-azure II. ×27.)

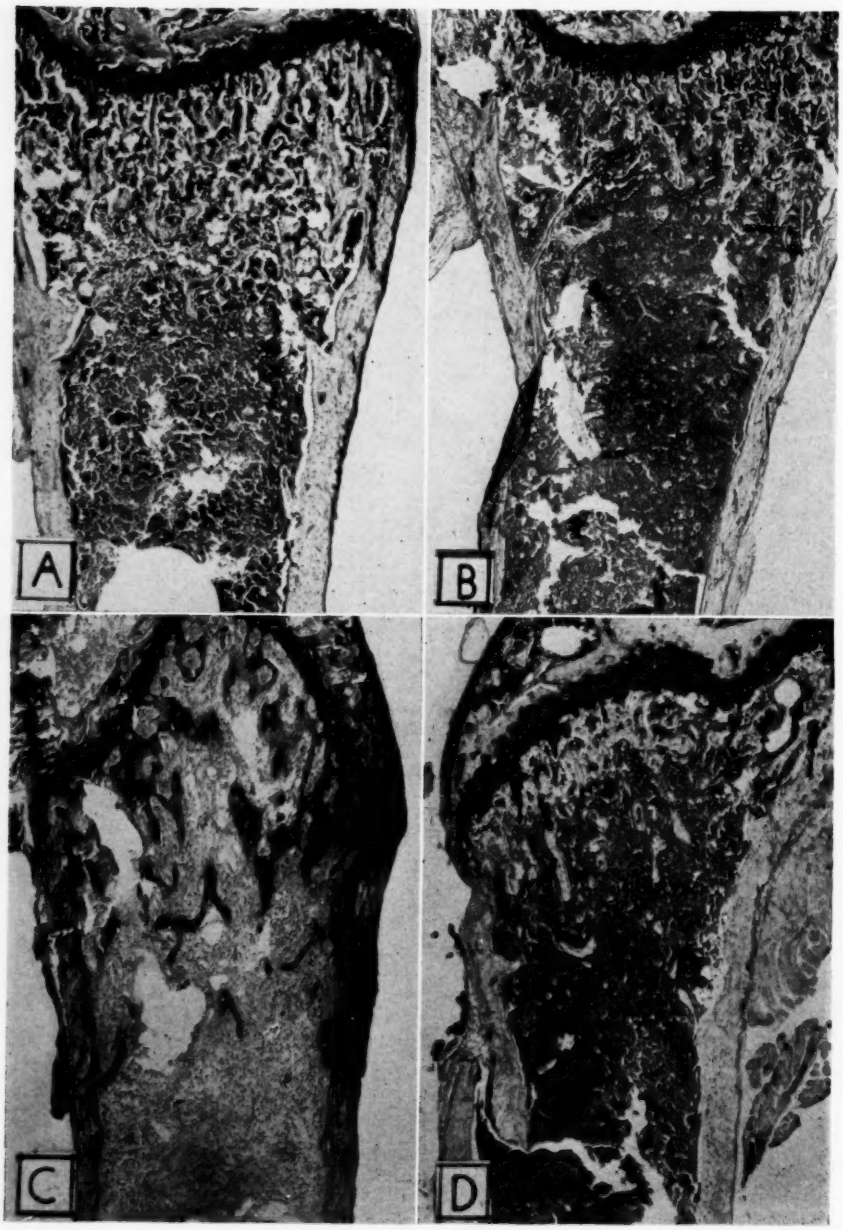

Fig. 11.—Femoral marrow of mice eight days after exposure to 1025 r whole body X-radiation. *A*, Control untreated (Group I); *B*, control operated only (Group II); *C*, whole body X-radiation without spleen protection (Group III); *D*, whole body X-radiation with spleen protection (Group IV).

Note that no hematopoietic cells are present and the epiphysis and metaphysis and part of diaphysis are replaced with atypical fibrous tissue in *C*, whereas a normal cellularity is present throughout the marrow space in *D*. (Hematoxylin-eosin-azure II. ×27.)

cells and widespread evidence of cell death (pyknosis, and so on). On the fourth day after irradiation, however, the bone marrow of this group (Group IV) was distinctly different from that of Group III (spleen unprotected) in that approximately 15 per cent of the normal marrow population persisted in the former, whereas, as already described, the femurs of Group III (spleen unprotected) animals were aplastic (Fig. 10 and Table II). On succeeding days this cellularity increased rapidly in extent and appeared normal on the eighth day and hyperplastic thereafter (Fig. 11). In only two of the femurs examined from the spleen-shielded group was "atypical fibrosis tissue" observed, and this in the metaphysis only at the four- and six-day intervals. The regeneration apparent even on the fourth day and increasing in extent at subsequent intervals appeared orderly. Mitoses were evident in all cell types. Transformation of reticular cells and bone cells to hemocytoblasts was prominent. Plasma cells were either relatively or absolutely increased in numbers in the marrow at the fourth, fifth, and sixth day intervals. Thereafter their numbers appeared to be in the normal range.

Bone marrow of animals which failed to survive: The foregoing descriptions are confined to animals sacrificed at definite intervals after irradiation. The bone marrow regeneration in animals from the irradiated groups which had died at various intervals after irradiation was essentially no different from that observed in animals from corresponding groups which were sacrificed at the same interval.

Lymphatic Tissue: The thymus, the lymph node chain at the root of the mesentery, the spleen, and a Peyer's patch were routinely taken for study.

Lymphatic tissue of animals exposed to 1025 r without lead protection of the spleen (Group III): The thymi of these irradiated animals (Group III) were markedly reduced in size in twenty-four hours and remained thus reduced in size through the thirteenth day which was the last interval of examination in this group. Microscopically some unphagocytosed cellular debris and debris-laden microphages were scattered about in both the medulla and cortex at the twenty-four hour interval. Practically no free cells remained at the two-day interval and only in an occasional specimen examined were foci of lymphocytes seen in the medulla and these only in occasional specimens from the sixth day or later. The shrunken thymus was thus composed of a cortex with a sheet of epithelial cells and a medulla composed of connective tissue with its attendant blood vessels (Fig. 12).

The lymph nodes from the root of the mesentery although reduced in size after irradiation were not as drastically so as the thymi, but like the thymi they did not resume normal size in animals sacrificed during the thirteen days after irradiation. Microscopically some residual particulate debris and a few lymphocytes with pyknotic nuclei and scattered debris-laden macrophages were evident twenty-four hours after irradiation. Free lymphocytes were absent from the cortex and medulla. The germinal centers were composed of closely packed reticular cells and the balance of the node was reduced to a reticular stroma. No evidence of regeneration occurred in the subsequent intervals; the remnants of germinal centers became smaller and more closely resembled clumps of epithelial tissue (Fig. 13 and Table III).

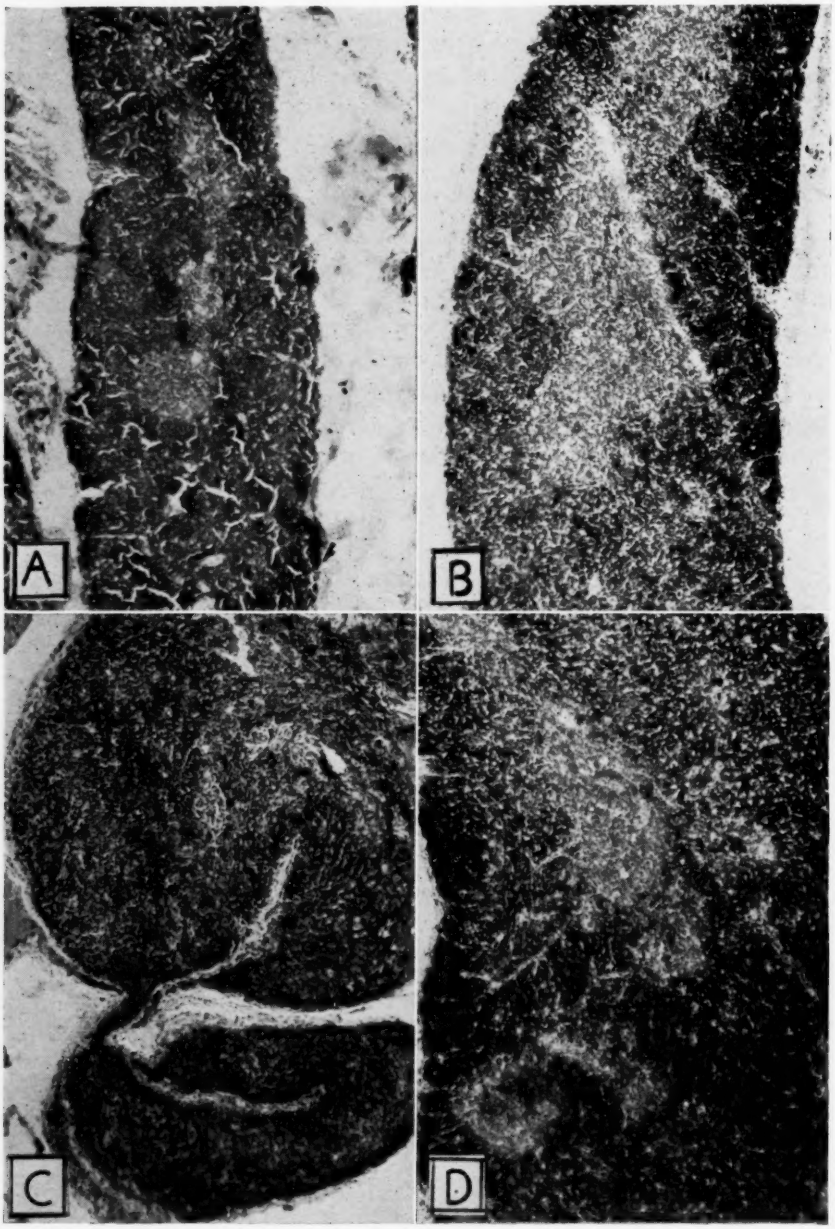

Fig. 12.—Thymi of mice eight days after exposure to 1025 r whole body X-radiation. A, Control untreated (Group I); *B*, control operated only (Group II); *C*, whole body X-radiation without spleen protection (Group III); *D*, whole body radiation with spleen protection (Group IV).

The thymus is reduced to a reticulum stroma and Hassall's corpuscles are prominent in C, whereas an essentially normal cellularity is present in *D*. (Hematoxylin-eosin-azure II. ×70.)

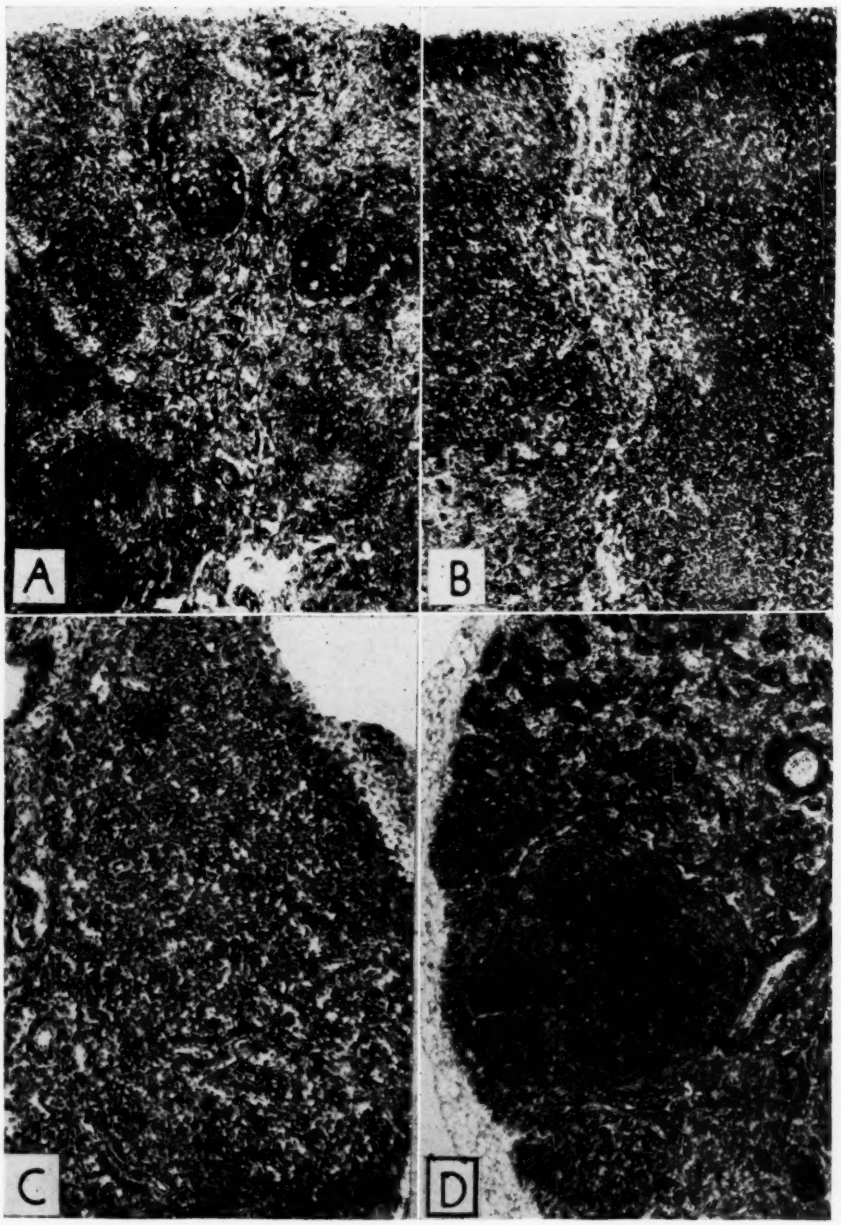

Fig. 13.—Mesenteric lymph nodes of mice eight days after exposure to 1025 r whole body X-radiation. *A*, Control untreated (Group I); *B*, control operated only (Group II); *C*, whole body X-radiation without spleen protection (Group III); *D*, whole body X-radiation with spleen protection (Group IV).

Note cell depletion, lack of nodules in *C* and normal cellularity with nodules in *D*. (Hematoxylin-eosin-azure II. ×70.)

The patches of Peyer were reduced in size and consequently were difficult to locate in many of the animals sacrificed at the various intervals after irradiation. In those examined microscopically, the findings were essentially similar to those observed in the lymph nodes.

The spleens were reduced in size twenty-four hours after irradiation by a factor of at least two and remained thus reduced in size at all subsequent intervals of examination. As noted previously,[2] an infarct invariably appeared in the distal tip of the spleen as a result of snipping a small vessel during the process of the surgical mobilization. This infarct involved from approximately one-sixth to one-fourth of the distal tip in Groups II, III, and IV. Twenty-four hours after irradiation the microscopic appearance of the spleen was strikingly different from controls. Evidence of lymphocyte death as well as death of erythroblasts, of myelocytes with nuclear degeneration, and of phagocytosis still persisted. The cellularity of both the white and red pulp was markedly reduced and only a few islands of erythroblasts existed in the red pulp and a few lymphocytes about the central arteriole in the nodules. At later intervals cellularity (free cells) was reduced to practically zero; the red pulp was composed at these intervals of a reticular stroma and connective tissue which gave the appearance of a sheet of fibrous scarring (Fig. 14). In some instances the nodules were reduced to an aggregate of reticular cells about the central arterioles or visible white pulp consisted of a few lymphocytes about the central arteriole. In other instances only the central arteriole remained. There was no evidence of regeneration of lymphatic tissues even at the thirteen-day interval, but in one of the two specimens available from this interval, foci of ectopic erythroblasts were present.

Lymphatic tissue of animals exposed to 1025 r with lead protection of the spleen (Group IV): Since the spleens of this group were lead-protected during irradiation, they will be described separately from other lymphatic tissues.

Some reduction of the size of the lymph nodes at the root of the mesentery and of the patches of Peyer was observed at the one-day interval after irradiation. Since these tissues were not weighed, only a qualitative estimation of change in size is possible. The lymph nodes and Peyer's patches were reduced by a factor of about two and studied at succeeding intervals. Both increased in size to reach approximately normal size on about the thirteenth to fifteenth day. The thymi of this group were likewise reduced to less than half size in twenty-four hours and were again approximately two thirds normal in size by the thirteenth to fifteenth day after irradiation.

Histologically the findings in the lymph nodes, thymi, and Peyer's patches in irradiated mice differed strikingly from those of Group III (unprotected spleens). As was observed in the bone marrow, the initial phase of destruction and phagocytosis during the first two days after irradiation was essentially identical in the two groups. Thereafter, the lymphatic tissue including the spleens of Group III (unprotected spleens) mice remained acellular without evidence of regeneration during the intervals studied through thirteen days. However, in the Group IV (spleen protected) animals the thymi, lymph nodes, and patches of Peyer had free lymphocytes on the fourth day in the stroma of

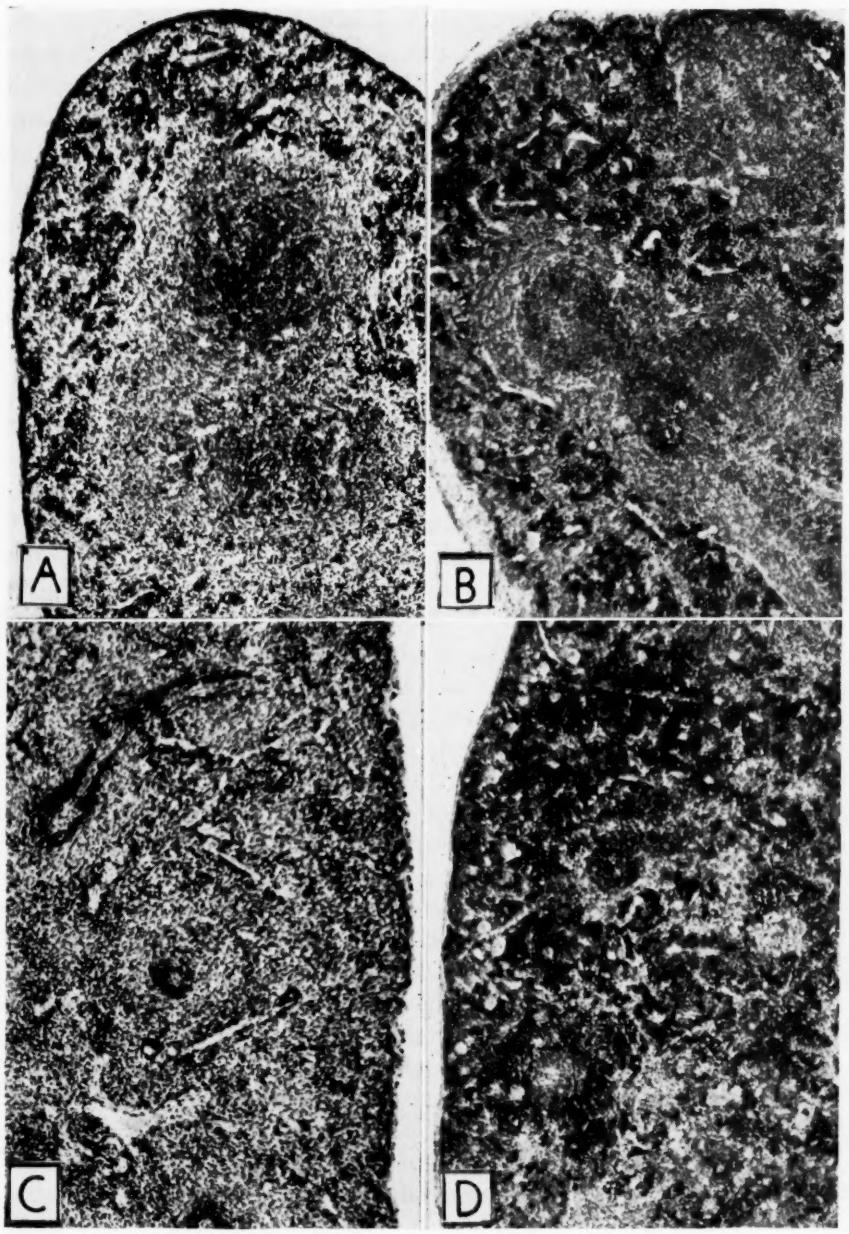

Fig. 14.—Spleens of mice eight days after exposure to 1025 r whole body X-radiation. *A*, Control untreated (Group I); *B*, control operated only (Group II); *C*, whole body X-radiation without spleen protection (Group III); *D*, whole body X-radiation with spleen protection (Group IV).

Note the depleted and atrophic white pulp, lack of ectopic blood formation, and prominence of reticulum in *C*, and the extreme degree of ectopic blood formation with virtual absence of white pulp and nodules in *D*. (Hematoxylin-eosin-azure II. ×70.)

both the cortex and medulla but the nodules were atrophic. At subsequent intervals studied, the cell population of the lymph nodes increased to the extent that in seven and eight days (though still somewhat reduced in size) an essentially normally populated tissue existed (Fig. 13 and Table III). Active nodules were apparent in both the lymph nodes and patches of Peyer. The cell population differed from the normal node in that in addition to lymphocytes a great increase in ectopic blood formation largely restricted to erythroblasts and myelocytes was everywhere to be found scattered about in these tissues between the nodules of the cortex and extending into the medulla. The thymi, though cellular at seven and eight days (Fig. 12), did not achieve normal cellularity until the twelfth to the fifteenth day interval. Ectopic erythro- and myelocytopoiesis were apparent in the thymi as early as eight days. It should be reemphasized at this point that without actually weighing these tissues any references to size of lymph nodes or thymi are qualitative since they represent estimates.

The spleen is considered separately since in this group (Group IV) it was lead-protected during irradiation. Actually little difference existed between the spleens of this group of animals that received 1025 r and the lead-protected spleens of animals exposed to 600 r as reported previously.[1] The spleens increased in size and in two to five days were approximately twice the normal size. By twenty-four hours the lymphocyte population of the white pulp had been reduced and ectopic erythropoietic tissue had increased. This increase in erythroblastic tissue as well as hemocytoblasts (stem cells) everywhere in the red pulp continued through the fourth day and concurrently the amount of white pulp decreased. In some specimens examined at this interval the white pulp consisted of a relatively sparse group of small and medium lymphocytes about the central arteriole (Fig. 14). The "invasion" of the white pulp by erythroblasts appeared to proceed as follows: islands of erythroblasts appeared in the white pulp—at later stages it appeared as though these islands had coalesced and the lymphocytes had disappeared except about the central arteriole. In other specimens groups of erythroblasts were seen actually beside the central arteriole of the white pulp even before any appreciable reduction in size of the white pulp aggregate had occurred. The source of these erythroblasts can only be speculated about. It may be that they simply wandered in from the red pulp or perhaps they represent transformation from reticular cells or from large lymphocytes. Beginning at the fourth day megakaryocytes were more prominent than at previous intervals throughout the red pulp and at about five days myelocytes were found scattered in relatively large numbers in the red pulp. At succeeding intervals megakaryocytes and myelocytes increased and erythroblasts decreased more or less proportionately. Beginning at about the eighth day, the white pulp began slowly to increase in size and reached approximately 30 to 40 per cent of normal by the thirteenth day. At subsequent intervals examined the intense ectopic blood formation continued in the spleen even though the bone marrow had returned to a normal cell population and ectopic hematopoiesis was increased in the lymph nodes.

Other Tissues: The liver, adrenal, small intestine, and kidney were routinely taken at autopsy. These have not been studied thoroughly yet. One observation of significance to this report, however, should be mentioned. Ectopic blood formation appeared in the liver of the animals which had lead protection of the spleen during irradiation but not in the animals without lead protection of the spleen during irradiation. The appearance of ectopic blood formation in this organ coincided with the reconstitution of the bone marrow (six to eight days). Ectopic blood formation was not observed in the kidney or adrenal of either of the irradiated groups.

DISCUSSION

These data raise interesting and fundamental questions regarding radiation injury which will be discussed below.

As was pointed out previously,[1, 2] the mechanism and the nature of the stimulus which induces ectopic erythropoiesis so rapidly in the lead-protected spleen of mice given 600 r whole body x-radiation are not clear since no anemia or other known stimulus of erythropoiesis appears to exist. No studies have been done, however, to determine whether oxygen saturation of the circulating hemoglobin is temporarily interfered with under these circumstances nor have we attempted to determine whether the blood flow in surgically mobilized spleen is temporarily reduced. The destruction of hematopoietic tissue may in itself produce the stimulus which is taken up immediately by the intact (lead-protected) spleen. Simmons and Jacobson[6] have shown in a preliminary experiment that in adrenalectomized mice an appreciable increase in ectopic erythrocytopoiesis occurs in the lead-protected spleens of mice given 600 r whole body x-radiation. These latter findings indicate that the stimulus for the rapid intensification of ectopic blood formation in the spleen probably neither originates in nor is mediated through the adrenal.

Even with a dose of 1025 r whole body x-radiation as shown in the data presented in this communication, the development of intense ectopic blood formation in the lead-protected spleen and the early recovery of the bone marrow and lymphatic tissue are sufficient to prevent an anemia of significance and to reduce the severity and shorten the duration of leucopenia and thrombocytopenia. As was originally observed in the experiment using 600 r referred to previously,[1] ectopic erythrocytopoiesis rapidly increases in the lead-protected spleen. Furthermore, erythrocytopoiesis appears to take precedence over lymphopoiesis as evidenced by the reduction in the size of the splenic white pulp which in turn keeps pace with the encroachment of erythrocytopoietic tissue. Ectopic erythropoiesis also appears to take precedence over ectopic granulo- and megakaryocytopoiesis. These latter cell types appear in the spleen in significant numbers only after three to four days, but they increase rapidly thereafter. Some reconstitution of lymphatic tissue in the lead-protected nonirradiated spleen begins at about the eighth day, which is coincident with the rapid phase of recovery in the bone marrow.

The return of the leucocytes (heterophiles and lymphocytes) of the peripheral blood to normal limits by the ninth day after irradiation in the animals

which had lead protection of the spleen correlates well with the histologic findings in the spleen, thymus, lymph nodes, and the bone marrow. At this interval (nine days) granulocytopoiesis has essentially reached a maximum in the spleen, and lymphopoiesis, as judged by the amount of white pulp, is beginning to increase. Erythrocytopoiesis has decreased at the ninth day stage compared with previous stages. We are reluctant to label the decrease in lymphatic tissue in the lead-protected spleen which begins to appear in the first twenty-four hours after radiation as an indirect effect of radiation. It appears rather to be reciprocally related to the increase and decrease in ectopic hematopoiesis in the spleen.

The survival of essentially 100 per cent of mice exposed to 600 r with lead protection of the surgically mobilized spleen, whereas 50 per cent regularly die with this dose if the spleen is not lead-protected, cannot yet be attributed solely to the rapid hematopoietic recovery in the former. After the administration of doses of 700, 800, and 900 r to mice without spleen protection, essentially no animals survive the twenty-eighth day period of observation; the survival is approximately 90 per cent, however, if the spleen is protected. On the other hand, after a dose of 1025 r, even with lead protection of the spleen, approximately 58 per cent fail to survive even though the hematologic findings in the peripheral blood and spleen are comparable to those of animals which were given 600 r and which also had lead protection of the spleen. This tends to indicate that up to a certain dose of radiation the hematopoietic function assumed by the spleen or perhaps other unknown functions of the spleen are capable of bridging the critical phase of radiation toxicity until such time as recovery ensues; whereas, with doses in the neighborhood of 1000 r or greater, damage to another system or systems is such that death of 58 per cent occurs in spite of the maintenance of essentially normal function of the hematopoietic system as judged by the hematologic findings in the peripheral blood and histologic study of hematopoietic tissues of the animals with lead protection of the spleen during irradiation.

Of great interest is the fact that, whereas destruction of the marrow, the thymus, and lymph nodes of both groups of irradiated mice was qualitatively similar on the first and second day after irradiation, a clear-cut difference existed among them thereafter. The marrows of the animals irradiated without spleen protection were essentially devoid of cells by the fourth day and no regeneration was apparent until the eleventh day after irradiation. On the other hand, in the irradiated animals which had lead-protected spleens during irradiation, free hematopoietic cells were present in the marrow on the fourth day and increased in abundance to the extent that the marrow was normal in cellularity by the seventh or eighth day. The thymus, the mesenteric lymph nodes, and the patches of Peyer (of the spleen-protected group) were reduced in size after irradiation but like the marrow were never depleted of free cells. These tissues approached normal size by the thirteenth to the fifteenth day after irradiation; active nodules were present in lymph nodes as early as six days and the general cellularity of lymph nodes and patches of Peyer was approximately normal by the eighth day. Hematopoiesis of all types except megakaryocytopoiesis was striking in

these lymphatic tissues. On the other hand, these tissues remained depleted of cells without evidence of regeneration in animals irradiated without spleen protection. In mice exposed to 600 r but with a technique otherwise identical to that described in this communication,[1] a less striking but nevertheless clear-cut difference in the rate of recovery existed between animals exposed to irradiation with lead protection of the spleen and animals irradiated without lead protection of the spleen. Thus, it would appear that this "suppressive effect" apparent in animals irradiated inclusive of the spleen is dependent on the dose.

The fact that the bone marrows, the thymi, the lymph nodes, and the patches of Peyer of the animals which had spleen protection were never depleted of free cells and returned to normal cellularity more quickly than did these tissues in animals without spleen protection is replete with possibilities for speculation. Several possibilities may be mentioned.

Irradiation of the spleen may alter the spleen to the extent that it functions as a pathologic organ and by some humoral mechanism suppresses hematopoietic regeneration during this critical phase of radiation toxicity.

Another and perhaps more likely possibility is that protection of the spleen simply provides the animal with a normal but hyperactive spleen which acts in some way to reduce "radiation toxicity" to the extent that the normal regenerative ability of hematopoietic tissue is permitted to proceed. However, the two irradiated groups closely paralleled one another as far as weight loss is concerned during the period in which the marrow, thymus, and lymph nodes of the spleen-protected group had already begun to regenerate, whereas no regeneration was apparent in the other group. Perhaps the normal spleen actively stimulates hematopoietic regeneration even in the presence of general radiation toxicity. Preliminary data from other experiments[8] indicate that lead protection of the spleen or the appendix of rabbits during exposure to 800 r whole body x-radiation is followed by a more rapid regeneration of the irradiated hematopoietic tissue than in animals irradiated without lead protection of the spleen or appendix. These findings indicate that lead protection of hematopoietic tissue from radiation rather than protection of the spleen specifically accounts for the earlier regeneration of the irradiated hematopoietic tissue and strongly suggests a hormonal or endocrine function either of these lymphatic tissues or of hematopoietic tissue generally. The so-called "indirect" therapeutic effect observed especially in myelogenous leucemia when the spleen alone is irradiated may be related to this same process. We have not yet completed our study of the effect of spleen protection on the recovery of other cells or tissues from radiation. Spleen protection may hasten regeneration of tissues other than those of the hematopoietic system. In fact, further studies may indicate that lead protection of any tissue may effect hematopoietic regeneration.

Another problem of interest in these experiments is concerned with the source of the cells in the bone marrow and lymphatic tissues of the animals with lead-protected spleens from which regeneration proceeds. The red pulp of the lead-protected spleens of these animals quickly becomes a site of intense ectopic blood formation and could be a source of any cell type from which colonization could occur. On the other hand, there is evidence of transition from bone cells

to hemocytoblasts (Downey's myeloblast) and reticular cell transformation to free hematopoietic cells within the marrow spaces and therefore it seems likely that if colonization occurs it is only one source of the cells which brings about regeneration.

In considering the mechanism of the production of anemia after irradiation, it has usually been assumed that the destruction of erythropoietic tissue in the marrow and such ectopic erythrocytopoietic tissue that exists elsewhere largely accounts for the anemia. Schwartz and co-workers[7] have indicated that urobilinogen excretion is increased after whole body irradiation in several species of mammals and they have therefore suggested that hemolysis plays some role in the production of anemia. Since destruction of hemoglobinated erythroblasts in the marrow occurs after irradiation, this source of urobilinogen must be considered in any calculation on the contribution of hemolysis to the anemia. After doses of whole body x-radiation in the LD_{50} range, the bone marrow of the mouse (600 r) and the rabbit (800 r) is largely destroyed. Histologic evidence of regeneration of erythrocytopoiesis begins in these species between the sixth and ninth day after irradiation but appreciable delivery of reticulocytes into the peripheral blood rarely begins before the ninth or tenth day after irradiation. The first manifestation of the anemia in the sense that hemoglobin, erythrocyte, and hematocrit values begin to fall is around the ninth day—the maximum anemia is usually manifest between the fourteenth and sixteenth day. Teleologically it would appear as though the body strives to maintain these values at a normal level until a functional regeneration has been established. In CF-1 female mice after exposure to doses of 600 r or even 1025 r whole body x-radiation no gross evidence of hemorrhage is apparent in the visceral tissues such as the intestine or in the subcutaneous tissue or muscles. Microscopic hemorrhage is seen, however, particularly in the lymph nodes, thymus, spleen, and bone marrow. The amount of microscopic hemorrhage is minimal and probably contributes in only a minor way to the anemia. With this in mind it seems likely that the anemia which appears after 1025 r cannot be accounted for only on the basis of destruction and inhibition of erythrocytopoiesis and hemorrhage unless one assumes that the life of the red cell in this species is ten days or less or unless microscopic hemorrhage is actually more extensive and widespread than our study would indicate. One might postulate, therefore, that hemolysis of erythrocytes plays a significant role in the development of anemia in the mice exposed to 1025 r and which had no lead protection of the spleen during irradiation. Proof of such a postulate must await more precise methods for studying hemolysis since pigment excretion studies would be incomplete in these mice which inevitably die within the first two weeks after irradiation. If, indeed, increased erythrocyte hemolysis occurred under the foregoing circumstances, it would be interesting for comparative purposes to determine whether hemolysis occurred also in mice given 1025 r with lead protection of the spleen during irradiation since no appreciable anemia becomes manifested in these latter animals.

SUMMARY AND CONCLUSIONS

Four groups of CF-1 female mice, 10 to 12 weeks of age, were used for study. Group I consisted of normal untreated controls. Groups II, III, and IV were

anesthetized with Nembutal and the spleens mobilized through a left upper quadrant incision with the main pedicle intact. The mice of Groups III and IV were given a dose of 1025 r whole body x-radiation but during irradiation the spleens of Group IV mice were placed in a lead chamber which reduced irradiation of this organ to a negligible dose. During irradiation the spleens of Group III mice were wrapped in sterile gauze or placed in a paraffin box which offered no appreciable shielding from irradiation. The spleens of all three groups were kept moist with physiologic saline during the period they were outside the abdominal cavity. After this procedure was completed, the spleens were returned to the abdominal cavity and the incisions sutured.

Hematologic, gross, and microscopic tissue studies and weight determinations were made on all four groups. The findings may be summarized as follows.

A severe anemia, leucopenia, thrombocytopenia, and reticulocytopenia occurred in mice without lead protection of the spleens that were exposed to 1025 r whole body x-radiation. No animals thus exposed survived beyond the thirteenth day after irradiation.

No significant anemia and only a transient leucopenia and thrombocytopenia occurred in mice given this dose (1025 r whole body x-radiation) if the spleen was surgically mobilized and lead-protected during irradiation. Forty-two per cent of these mice survived beyond the twenty-eight days of observation.

The lymphatic tissues (thymi, lymph nodes, and spleens) of mice *without lead protection of the spleen* during irradiation were reduced to a reticulum stroma and no recovery was apparent during the thirteen days of observation.

The lymphatic tissues of mice *with lead protection of the spleens* during irradiation never became depleted of free cells and resumed an essentially normal cellularity by the seventh or eighth day after irradiation. Ectopic erythrocytopoiesis and granulocytopoiesis were increased remarkably in these lymphatic tissues by the eighth day after irradiation.

Hematopoiesis in the bone marrows of mice *without lead protection of the spleen* during irradiation was destroyed; the bone marrow remained thus depopulated through the tenth day after irradiation, and only beginning regeneration was apparent on the eleventh day after irradiation. Atypical fibrous tissue involving chiefly the metaphysis and epiphysis but occasionally involving the diaphysis as well was prominent at all intervals of study from the fourth day on.

The bone marrows of mice with lead protection of the spleen during irradiation were never depleted of free hematopoietic cells and were normal in cellularity at seven or eight days and hyperplastic thereafter. Atypical fibrous tissue was inconspicuous in the bone marrow of this group.

A marked increase in ectopic blood formation in the lead-protected spleen of the animals given 1025 r whole body x-radiation occurred. This increase was increased steadily through five to six days. The initial increase was largely erythroblastic but beginning between the fourth and sixth days an increase in megakaryocytic and granulocytic precursors was apparent. Concurrent with increased ectopic erythrocytopoiesis in the spleen, the splenic white pulp decreased in amount.

Undoubtedly, this rapid development of ectopic blood formation in the lead-protected spleen and the early recovery of the bone marrow and lymphatic tissues accounts for the fact that no anemia and only a modest and transient leucopenia and thrombocytopenia occurred.

The mean weight of mice irradiated with spleen protection and of mice irradiated without spleen protection fell more or less equally during the first five days following exposure. Thereafter, the mean weight of the mice without spleen protection continued to fall until death of all animals occurred, whereas the mean weight of mice which had spleen protection began to rise and reached the normal range by the eighteenth day.

Tentative interpretations based on the foregoing findings are related in the discussion.

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THE IMMUNOLOGIC RESPONSE OF CHILDREN TO THE INJECTION OF CHICK EMBRYO PROPAGATED MUMPS VIRUS VACCINE, AS MEASURED BY THE COMPLEMENT FIXATION TEST

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THE experimental transmission of mumps to monkeys by Johnson and Goodpasture[1-4] and more recently the adaptation of the mumps virus to the developing chick embryo by Habel,[5] Levens and Enders,[6] and Beveridge and co-workers[7] have greatly widened the field for investigation and increased the possibilities for eventual development of a prophylactic vaccine.

Various attempts to vaccinate against mumps have been made. Johnson and Goodpasture,[3] in one method, sprayed material containing monkey parotid gland live virus into the nose and oral cavity in their attempt to immunize monkeys. Enders and co-workers[8-10] obtained encouraging results with inactivated Formolized and alum-precipitated Formolized suspensions of infected monkey parotid gland, as well as with egg-adapted live virus of lowered virulence in the vaccination of monkeys and children. Habel[11] employed an ether-killed, egg-adapted virus vaccine. Beveridge and Lind[12, 13] inactivated amniotic and allantoic fluid virus with 0.1 per cent Formalin and found an appreciable increase in the hemagglutination-inhibition titer of vaccinated subjects. Attempts also have been made to determine resistance or susceptibility to mumps by intradermal inoculation of inactivated mumps virus.[5, 14]

The adaptation of mumps virus to hosts other than man and the monkey has been successful in one instance only—when Wollstein[15-17] reproduced parotitis, orchitis, and meningitis in cats. In this laboratory, attempts to adapt the virus to guinea pigs, hamsters, mice, cotton rats, and rabbits by various routes of inoculation gave only negative results. This marked host-specificity of the mumps virus complicates any study of the prophylactic value of a vaccine. Although the animals just cited are not susceptible to infection, they do respond immunologically to the injection of live or killed virus and their response can be measured either by complement fixation or by the virus neutralization test in the developing chick embryo. Having obtained sera of high complement-fixing titers from guinea pigs and hamsters, the serologic method was adopted for the determination of the immunologic value of a vaccine.

The report which follows presents data on the skin sensitivity and the immunologic response of children before and after injection of a Formalin-killed, egg-adapted, and partially purified and concentrated mumps virus vaccine.

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MATERIAL AND METHODS

Mumps Virus.—An egg-adapted strain of mumps virus in the twenty-second allantoic sac passage was kindly supplied by Dr. Karl Habel of the National Institutes of Health. This strain was propagated further in 8-day-old embryos, incubated at 35° C. for seven days, by inoculation of 0.2 ml. of a 10^{-3} dilution of infected allantoic fluid into the allantoic sac through a hole drilled in the shell over the air sac. This dilution, found by previous egg titrations to contain approximately 10,000 MID, gave a high yield of virus, as measured by the agglutination of chicken red blood cells. Titration of the virus in vitro was made by the Salk method.[18]

Preparation of Vaccine.—Eight-day-old eggs were inoculated as described and all dead embryos, which seldom exceeded 10 per cent of total eggs inoculated, were discarded up to the seventh day of incubation and only the surviving embryos were harvested. The eggs were first chilled overnight at 4° C. and then processed in a manner essentially similar to that used for production of influenza vaccine by the Sharples centrifugation method as modified for use in this laboratory.[19-22] The hemagglutination titer of freshly harvested allantoic fluid usually varied from 1:400 to 1:800. After centrifuging, the hemagglutination titer of the concentrated vaccine varied from 1:1,600 to 1:2,240 and sometimes reached titers as high as 1:2,800 to 1:3,200. However, the hemagglutination titer falls quite rapidly on standing at 4° C. Formalin and Merthiolate were then added to the supernate to give a final concentration of 1:5,000 and 1:10,000 respectively. The vaccine was allowed to stand for twenty-four to forty-eight hours at 4° C. before sterility tests were made. Its total nitrogen content varied between 0.1 mg. and 0.15 mg. per milliliter. After standing at 4° C. for three to four days, no live virus could be demonstrated by inoculation and subculture in 8-day-old chick embryos. Guinea pigs given two subcutaneous injections of 0.5 ml. each of the concentrated mumps vaccine spaced a week apart, and bled the third week, showed an appreciable antibody titer as measured by the complement fixation test. For example, four guinea pigs vaccinated with batch No. 7-1094-3A, with which the vaccination of children was carried out, showed complement-fixing titers of 1:64, 1:256, 1:128, and 1:64. In a neutralization test in eggs, the guinea pig serum with a complement-fixing titer of 1:256 neutralized approximately 10,000 MID of virus at the serum dilution of 1:640.

Complement-Fixing Antigen.—The complement-fixing antigen routinely used for the testing of all sera was a fivefold concentrated infected allantoic fluid which contained 0.1 per cent Formalin. It remained stable for several months when stored at 4° C.

Skin Test Antigen.—The skin test antigen used throughout this study consisted of the concentrated vaccine diluted 1:10 with 0.1M phosphate buffer, pH 7.0.

Serologic Methods.—All sera were inactivated at 56° C. for thirty minutes, and serial twofold dilutions, made in buffered saline,[23] ranging from 1:4 to 1:256 were examined by an overnight icebox complement-fixation technique similar to that described by Kolmer and Boerner[24] as modified for general use in this laboratory for the viral and rickettsial serologic studies.[25] Two *full* units of complement were used.

The unit of mumps antigen was first determined in the presence of 4 to 8 units of a known mumps immune serum and two *antigenic* units were used in testing of all sera.

RESULTS

A group of 125 healthy children at a childrens' home, comprising an equal number of boys and girls between 9 and 15 years of age, were vaccinated by two different routes. Most of these children came from crowded sections of New York City. Some remembered having had mumps in the past and a few had had the disease during the previous two or three years at the home, but on the whole no accurate history could be secured. No cases of mumps were known to have occurred at the home for several months prior to the vaccination. Except for an erythema at the site of inoculation, sometimes accompanied by a slight

edema which disappeared within forty-eight hours following injection, no other reactions, local or general, were observed as a result of vaccination.

The children were divided into five groups of twenty-five persons each (Groups I to V). Age distribution was comparable in each group. The prevaccination bleeding, skin testing, and the first injections of vaccine were carried out on the same day. Children in Groups I and II received 0.1 ml. intracutaneously, whereas the children in Groups III and IV were given 0.5 ml. subcutaneously. Group V served as unvaccinated control, but blood samples were taken and skin tests were also performed on these children. Two weeks later a second dose of vaccine, in the same amounts and by the same routes, was given to the children in Groups II and IV, and at three weeks blood samples were secured from the children in all five groups. At seven weeks a booster dose of vaccine, in identical amounts and by the same routes as given in the first injection, was administered to the same children in Groups I through IV. At the ninth postvaccination week blood samples were obtained again from all of the children (including Group V). Approximately nine months after the vaccination was completed (or one year from the first inoculation) an additional blood sample was obtained from most, but not all, of the children.

This schedule was based on previous observations of the immunologic response of guinea pigs given varying doses of vaccine at different intervals. It was intended to establish the skin sensitivity status of the children, as well as to evaluate the response to a single injection followed by a booster dose at seven weeks as compared with two injections given two weeks apart and similarly followed by a booster dose. The choice of a seven-week interval for the booster dose was based on the observations of complement-fixing titers in sera of individual guinea pigs which showed that the antibodies reached a low level or disappeared completely at about that time, and a booster dose caused a considerable increase in titer as early as one week after its injection. Since the skin test material in itself induces an increase in complement-fixing titer, as already observed by Enders and associates,[14] blood samples from the control group, also skin tested with the same amount of material as the four other groups, were tested to determine the extent of the increase.

Skin testing was carried out by inoculating intracutaneously on the anterior surface of the right forearm 0.1 ml. of a 1:10 dilution of vaccine. Readings of the smallest and largest diameters of erythema were made at twenty-four and

TABLE I. CORRELATION OF SKIN TEST REACTIONS TO COMPLEMENT-FIXING TITERS OF PREVACCINATION BLOOD SAMPLES

C.F. TITER	SKIN TEST OVER 15 MM.	SKIN TEST BETWEEN 15 AND 10 MM.	SKIN TEST BELOW 10 MM.	TOTAL C.F.
<1:4	7		2	9
1:4	12		1	13
1:8	38	7	8	53
1:16	32	6	1	39
1:32	9			9
1:64	1			1
1:128	1			1
Total skin test	100	13	12	125

forty-eight hours after injection, and mean values were calculated on that basis. As recommended by Enders and co-workers,[14] three categories were employed: definite positives (more than 15 mm. diameter), doubtfuls (between 10 mm. and 15 mm.), and definite negatives (less than 10 mm.). Skin test measurements, distributed according to titers obtained by the complement fixation test on the prevaccination sera, are summarized in Table I.

Calculation of percentages of positives and negatives obtained by the skin test and the complement fixation test can be summarized as follows:

	SKIN TEST		COMPLEMENT FIXATION	
	NUMBER	PER CENT	NUMBER	PER CENT
Positive	100	80.0	116	92.8
Doubtful	13	10.4		
Negative	12	9.6	9	7.2

Evaluation on another basis gave:

	NUMBER	PER CENT
Skin test pos., C.F. pos.	106	84.8
Skin test pos., C.F. neg.	7	5.6
Skin test neg., C.F. pos.	10	8.0
Skin test neg., C.F. neg.	2	1.6

Normal allantoic fluid and Formalin controls were not used in this test. However, according to Ratner and Untracht,[26] reactors to egg proteins in a general population do not exceed 0.5 per cent and this should not materially modify the figures obtained. As can be seen from the foregoing summary, by both the skin test and the complement fixation test 84.8 per cent of the total number of individuals would be classified in Enders'[14] immune category. Only two of the 125 children showed susceptibility by both tests. This high percentage of immune individuals might be expected within a group of children of that age range originating from crowded parts of New York City where the chance of infection at an early age is highly probable.

The prevaccination serum samples of most of the individuals from the five groups showed appreciable complement-fixing titers with mumps antigen, thus indicating past history of mumps, either clinical or inapparent. Some of the children who showed no significant titer before vaccination gave high titers after a single injection of vaccine, indicating a probable past history of the disease with complete disappearance of complement-fixing antibodies and an anamnestic response to a single dose of vaccine.[27] Two children of Group I remained negative after the dose of vaccine and showed measurable titers only after the booster injection given seven weeks later. These children may be regarded as the only true susceptible members of the whole group, which seems to indicate the advisability of repeated injections for the vaccination of true susceptible persons.

Geometric mean titers were calculated for each group and are presented in Fig. 1. In this figure it is seen that in the third-week bleedings greater C.F. titers were obtained in the four vaccinated groups than in the control group, and the vaccinated groups show still greater titers after the booster injection, whereas the control group shows a decrease in titer at that time. One year after vaccination the titers in all five groups showed a considerable diminution but

the titers in the four vaccinated groups still remained greater than in the control group. However, no appreciable difference in antibody titers was found in any of the vaccinated groups at the end of one year.

The rather large percentage of children in all five groups showing appreciable prevaccination complement fixation titers and giving an immune response to the injection of skin test antigen complicates the analysis of the results. A similar situation is met with in influenza, in which most individuals possess demonstrable prevaccination titers.

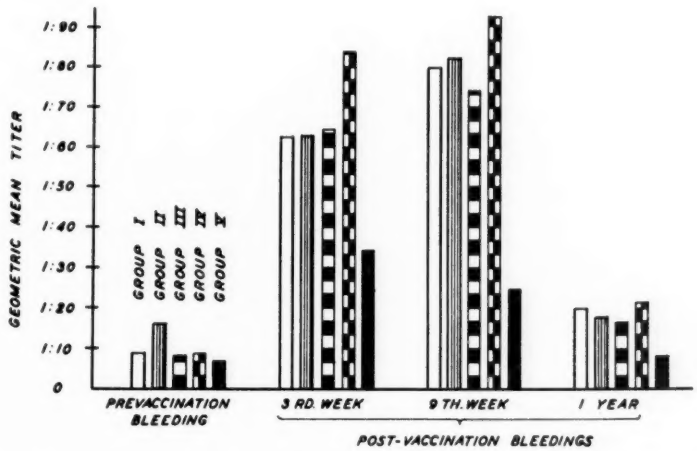

Fig. 1.—Comparative geometric mean complement-fixing titers in blood samples of various groups of children vaccinated against mumps.

The establishment of an arbitrary antibody level as a base line would greatly assist in making comparison of results before and after vaccination in such cases. Unit-gains as well as unit-fold increases could then be calculated on that basis and a clearer picture could be obtained in a comparative study of the immunologic response of different vaccinated groups. In this particular study a 1:4 complement-fixing titer was arbitrarily chosen as one antibody unit. Table II gives a summary of the results tabulated in complement-fixing unit-gains and in complement-fixing unit-fold increases. No appreciably significant differences are found in any of the vaccinated groups at the end of one year, although Group IV in all instances showed a slightly higher antibody level. The control group (Group V) continued to show low titers.

DISCUSSION

The data presented in this paper show that the vaccination of children with a concentrated mumps virus vaccine induces a serologic response which can be measured by the complement fixation test, using a concentrated viral suspension as antigen. Two subcutaneous injections of 0.5 ml., given two weeks apart, seem to induce a better response than two injections of 0.1 ml. given intracutaneously similarly spaced, or single injections of 0.1 or 0.5 milliliter. The need for repeated injections was particularly demonstrated by the two

TABLE II. CORRELATION OF C.F. UNIT-GAINS AND C.F. UNIT-FOLD INCREASES FOR THE FOUR VACCINATED GROUPS AND THE UNVACCINATED CONTROL GROUP

	GROUP I			GROUP II			GROUP III			GROUP IV			GROUP V		
	2 INJECTIONS 0.1 ML. INTRACUTANEOUSLY			3 INJECTIONS 0.1 ML. INTRACUTANEOUSLY			2 INJECTIONS 0.5 ML. SUBCUTANEOUSLY			3 INJECTIONS 0.5 ML. SUBCUTANEOUSLY			UNVACCINATED CONTROLS		
BLOOD SAMPLES	C.F. UNITS	UNIT-GAIN	UNIT-FOLD INCREASE	C.F. UNITS	UNIT-GAIN	UNIT-FOLD INCREASE	C.F. UNITS	UNIT-GAIN	UNIT-FOLD INCREASE	C.F. UNITS	UNIT-GAIN	UNIT-FOLD INCREASE	C.F. UNITS	UNIT-GAIN	UNIT-FOLD INCREASE
Prevac.	2.2	—	—	3.8	—	—	2.1	—	—	2.2	—	—	1.7	—	—
3rd. wk.	15.5	13.3	7.0	15.5	11.7	4.1	15.9	13.8	7.6	20.9	18.7	9.5	8.7	7.0	5.1
9th wk.	19.9	17.7	9.0	20.5	16.7	5.4	18.5	16.4	8.8	22.9	20.7	10.4	6.0	4.3	3.5
1 yr.	5.0	2.8	2.3	4.5	0.7	1.2	4.1	2.0	2.0	5.2	3.0	2.4	2.1	0.4	1.2

true susceptible subjects of Group I who had no C.F. titers before vaccination and remained negative after one 0.1 ml. dose intracutaneously, as shown by the results of the third-week bleedings. However, after the seventh-week booster dose, the ninth-week bleedings showed respectively 1:16 and 1:32 C.F. titers. At the end of one year the titer of the first child was practically back to zero; the second child had left the home and no serum sample could be secured.

The skin sensitivity test with vaccine diluted 1:10 applied to the same children indicated that 90.4 per cent were immune to mumps, according to the criterion of Enders and co-workers,[14] whereas 92.8 per cent had complement-fixing antibodies in the prevaccination blood samples; 84.8 per cent apparently were immune by both methods. The close correlation obtained by these two methods would indicate that either one could be used satisfactorily in a survey for the determination of susceptibility to mumps, but from the practical standpoint skin testing is a more convenient procedure.

Who should receive mumps vaccine? Because of the fact that mumps is a widespread disease, few individuals fail to contract it during childhood in urban areas. The disease in children usually lacks the severity and complicating sequelae seen in adults. One attack of mumps appears to immunize against subsequent infections, but second attacks have been reported.[28] Therefore, it does not seem justified that indiscriminate vaccination of children should be recommended, unless they are housed in large groups in institutions and vaccination is repeated at regular intervals to keep the antibody titer at an adequate level at all times.

According to the statistical data presented by Wesselhoeft,[29] approximately 80 per cent of the urban population by the age of 17 has contracted the disease. This leaves 20 per cent who are susceptible upon reaching adulthood. Furthermore, in rural populations more adults are susceptible than in urban areas, and vaccination could be used to good advantage even if limited to adults in preventing epidemics among them. The most obvious communities of adults to be vaccinated would be those who live in close contact for any length of time, such as military personnel, college students, nurses, physicians, and patients confined to hospitals for long periods of time. Skin testing could be used to indicate most of the susceptible persons who then could be vaccinated, but it would be simpler and less time consuming if the entire population of an institution, barracks, or a college campus were vaccinated. Our observations, first made on guinea pigs and then on human subjects, indicate that at least two injections of vaccine, whatever the dose employed, administered at two- to three-week intervals, are necessary to give rise to significant serologic titers in true susceptible persons. Since mumps is a seasonal disease occurring mainly in late fall, winter, and early spring, the first injection could be given during the month of June (in the case of students, just before summer recess) and the second dose in September or October. Thus, the required antibody level would be induced in time to afford protection during the epidemic mumps season. A one- to three-week interval between the first and second injection also could be used satisfactorily.

SUMMARY

A Formalin-inactivated mumps virus vaccine was prepared by concentrating infected allantoic fluid by means of the Sharples centrifuge.

The vaccine material, in a dilution of 1:10, was used satisfactorily for skin testing of 125 individuals.

One hundred of the same individuals were vaccinated, using different doses at different time intervals, and an appreciable increase in antibody levels was observed, as judged by complement fixation tests.

Skin testing and complement fixation seem to be of comparable value for the determination of susceptibility.

A tentative schedule of vaccination against mumps is presented.

The authors wish to acknowledge the able technical assistance given by Miss Violet Barberis in the performance of the serologic tests.

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LABORATORY METHODS

STUDIES ON THE STABILITY OF THE HISTOPLASMIN COLLODION AGGLUTINATION TEST

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PREVIOUS publications[1-3] from this laboratory have demonstrated the applicability of the collodion agglutination technique as a valuable adjunct to the complement fixation test[4-6] for histoplasmosis. Histoplasmin by virtue of its clarity and antigenicity adapts itself readily to use with collodion particles. Norden[7] has described recently a comparable hemagglutination test using histoplasmin since "the preparation of collodion particles is very difficult." Havens and Lloyd[8] have devised a shorter method for the preparation of these particles which remain stable for seven to fourteen days. In a laboratory doing daily routine testings, the marked stability of the particles as used in our studies and the simplicity of performing the test may result in a far greater economy of time than the methods referred to.[7, 8] The following data are presented to emphasize the stability, simplicity, and repeatability of the histoplasmin collodion agglutination test.

MATERIALS AND METHODS

The collodion particles are prepared by the method of Cavelti[9] as noted in a previous report.[1] However, instead of storing the particles in a heavy stock suspension which requires dilution each day before testing, the material is maintained in the form of a "working suspension." The latter is adjusted to match a No. 8 rather than the No. 2 MacFarland nephelometer in order to obtain heavier macroscopically visible flocculation. This suspension allows a light transmission of 11 per cent at 440 mμ on the Coleman spectrophotometer. The "working suspension" is stored in a round rubber-stoppered 3-liter flask in the dark at room temperature. There is very little tendency for the particles to settle on standing and agitation in a rotary motion before use results in a homogeneous suspension which is of the same concentration on repeated test days.

An equal quantity of the optimal dilution of histoplasmin as determined by previous titration in the presence of positive sera is added to the collodion particles and the test conducted as originally described.[1] This previously determined dilution rarely varies from the same sample of histoplasmin and periodic checks reveal the stability of the antigen.

RESULTS

1. Reproducibility of Results.—As in any serologic test the repeatability of results is an important criterion of the procedure's value. Periodic trial runs are made wherein multiple samples of the same sera are submitted to the technician setting up the tests and labeled in such a manner that only the

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referee knows the contents of each tube. The results of such an experiment are listed in Table I in which multiple specimens of rabbit sera of varying antibody levels were included in a random group of several hundred. The tests were performed by four persons and minor differences in results were to be expected. However, as a whole, the results as can be seen in Table I showed a very high degree of correlation. As noted in our previous report,[1] normal rabbit sera did not react with the sensitized particles. Since that time it has been only the rare normal rabbit serum which will react and then only in low intensity (1+ or 2+) and titer (1:5 to 1:10). This is in contrast to the results with "normal" human sera where about 25 per cent[10] yield 1+ to 2+ reactions in low titer. Thus whereas with rabbit sera one may consider reactions less than 4+ in intensity as significant of an antibody response, with human sera a 4+ reaction must be obtained before significance is assessed.

TABLE I. REPEATABILITY OF RESULTS IN THE COLLODION AGGLUTINATION TEST USING NORMAL AND IMMUNE RABBIT SERA AND AN OPTIMAL DILUTION (1:200) OF HISTOPLASMIN (H-40)

	RECIPROCAL OF TITER								RECIPROCAL OF TITER						
	5	10	20	40	80	160	320		5	10	20	40	80	160
Specimen A: Complement Fixation Negative*								*Specimen D: Complement Fixation at 1:80*						
980	–	–	–	–	–	–	–	770	3+	3+	2+	2+	±	–
830	–	–	–	–	–	–	–	464	4+	4+	3+	1+	–	–
228	–	–	–	–	–	–	–	832	3+	4+	3+	2+	±	–
21	–	–	–	–	–	–	–	659	2+	3+	2+	1+	±	–
925	–	–	–	–	–	–	–	87	3+	3+	2+	1+	±	–
216	–	–	–	–	–	–	–	157	4+	4+	3+	1+	±	–
894	–	–	–	–	–	–	–	252	1+	4+	2+	1+	±	–
585	–	–	–	–	–	–	–	407	4+	4+	3+	1+	±	–
969	–	–	–	–	–	–	–	269	4+	4+	1+	1+	–	–
239	–	–	–	–	–	–	–	491	4+	4+	4+	3+	±	–
Specimen B: Complement Fixation Negative								*Speciment E: Complement Fixation at 1:40*						
851	–	–	–	–	–	–	–	652	2+	1+	1+	–	–	–
896	–	–	–	–	–	–	–	222	1+	1+	1+	–	–	–
660	±	–	–	–	–	–	–	778	1+	1+	1+	–	–	–
449	–	–	–	–	–	–	–	44	1+	1+	±	–	–	–
73	–	–	–	–	–	–	–	283	1+	1+	1+	–	–	–
635	±	–	–	–	–	–	–	318	1+	1+	±	–	–	–
5	–	–	–	–	–	–	–	463	4+	2+	2+	±	–	–
225	–	–	–	–	–	–	–	656	1+	1+	±	–	–	–
38	–	–	–	–	–	–	–	705	1+	1+	1+	–	–	–
681	–	–	–	–	–	–	–							
Specimen C: Complement Fixation at 1:320								*Specimen F: Complement Fixation at 1:40*						
130	4+	4+	4+	4+	3+	1+	–	53	4+	3+	1+	±	–	–
734	4+	4+	4+	4+	4+	1+	–	595	4+	4+	3+	±	–	–
64	4+	4+	4+	4+	4+	1+	–	552	4+	3+	1+	±	–	–
104	4+	4+	4+	4+	4+	2+	–	101	4+	4+	1+	1+	–	–
19	4+	4+	4+	4+	4+	3+	–	292	4+	4+	2+	±	–	–

*Every number under each specimen represents a sample of the respective serum specimen.

The data in Table I present the results obtained with forty-nine separate samples representing sera from six different rabbits (A to F). The corresponding complement fixation data are recorded for comparative purposes.

2. Stability of Collodion Suspensions.—In our experience 20 to 25 Gm. of dried collodion are obtained from a 1 lb. bottle of collodion (Merck). Ten grams will usually yield about 3 liters of "working suspension." The re-

mainder of the dried material may be stored in an evacuated desiccator at 37° C. indefinitely and may be used for future preparations. Since the collodion suspension is mixed with an equal quantity of histoplasmin before each run, a potential volume of 6 liters of antigen is available from the original

TABLE II. COMPARATIVE RESULTS IN THE COLLODION AGGLUTINATION TEST USING THREE DIFFERENT LOTS OF COLLODION PARTICLES AND POSITIVE AND NEGATIVE HUMAN AND RABBIT SERA

SERA	COLLODION LOT*	SERUM DILUTIONS 1:5	1:10	1:20	1:40	1:80	1:160
Positive human sera							
1. 869	O	4+	4+	3+	2+	±	–
	M	4+	3+	1+	–	–	–
	N	4+	4+	2+	1+	±	–
2. 886	O	4+	4+	4+	3+	1+	±
	M	4+	4+	4+	2+	±	–
	N	4+	4+	4+	3+	2+	±
3. 799	O	4+	4+	4+	1+	–	–
	M	4+	3+	3+	–	–	–
	N	4+	4+	1+	–	–	–
4. 887	O	4+	3+	1+	±	–	–
	M	1+	1+	–	–	–	–
	N	4+	4+	3+	1+	–	–
5. 828	O	4+	4+	4+	2+	±	–
	M	4+	4+	1+	±	–	–
	N	4+	2+	2+	–	–	–
6. 803	O	4+	4+	4+	4+	2+	1+
	M	4+	4+	4+	4+	2+	1+
	N	4+	4+	4+	4+	4+	2+
7. 731	O	4+	3+	2+	–	–	–
	M	3+	1+	±	–	–	–
	N	4+	4+	1+	–	–	–
Negative Human sera							
8.-10. 862	O	–	–	–	–	–	–
863	M	–	–	–	–	–	–
819	N	–	–	–	–	–	–
11. 883	O	±	±	–	–	–	–
	M	1+	2+	1+	–	–	–
	N	–	–	–	–	–	–
Positive rabbit sera							
12. 3662	O	4+	4+	1+	±	±	–
	M	4+	4+	3+	1+	–	–
	N	4+	4+	1+	1+	±	–
13. 3819	O	4+	4+	3+	1+	±	–
	M	4+	4+	4+	1+	±	–
	N	4+	4+	3+	1+	±	–
14. 3820	O	3+	±	–	–	–	–
	M	3+	1+	±	–	–	–
	N	3+	±	–	–	–	–
Blastomyces rabbit sera							
15. 3817	O	1+	±	–	–	–	–
	M	1+	1+	±	–	–	–
	N	1+	±	–	–	–	–
16. 3821	O	2+	±	–	–	–	–
	M	±	±	–	–	–	–
	N	±	±	–	–	–	–
Normal rabbit sera							
17, 18. 3203	O	–	–	–	–	–	–
3204	M	–	–	–	–	–	–
	N	–	–	–	–	–	–

*O, M, and N represent lots of collodion 14 months, 3 months, and 3 weeks old respectively.

10 Gm. of dried collodion. The amount of sensitized particles used in each tube is 0.5 ml. so that approximately 24,000 individual tube tests are theoretically possible. Of course, with the discarded unused antigen that there may be from day to day, this number of tests is not obtained, but one can readily observe that a considerable number of serum specimens can be studied with a single lot of collodion. Since we routinely use six serial dilutions for each serum specimen (1:5 to 1:160) in preliminary screenings, the maximum number of sera that could be studied under these conditions approaches one-sixth the total volume of 24,000 ml. or 4,000. One would therefore be concerned with the keeping qualities of the collodion so that the maximum use could be made of the material. Although we rarely have had occasion to use particles over 6 months old, the material has been found to be remarkably stable over periods even greater than this. The data as shown in Table II demonstrate the results obtained on the same sera with three different lots of collodion particles. At the time of testing, Lots O and M which were 14 and 3 months old respectively were compared with the current lot, N, which was 3 weeks old. The results obtained here with positive human and rabbit sera showed a rather close correlation in test results. Since the 4+ reaction is considered positive with human sera and 3+ suspicious or doubtful, the only divergent results were noted with Lot M on Sera 887 and 731 where 1+ and 3+ reactions were obtained in comparison to 4+ reactions with the fresh lot. On the other hand, negative results were obtained with all three lots on the four representative negative sera listed indicating that false positives do not occur with the old preparations. The latter results have been observed repeatedly on large groups of normal sera from both human and rabbit sources.

Sera 3662, 3819, and 3820 were obtained from rabbits immunized with *Histoplasma capsulatum* and represent different degrees of antibody titers. Rabbits 3817 and 3821 were immunized with the cross reacting *Blastomyces dermatitidis* organism while Sera 3203 and 3204 are representative of the results obtained with normal rabbit sera. The results observed with the rabbit sera using different lots of collodion were comparable with those obtained with human sera.

These data therefore indicate that the collodion particles as used in this laboratory are relatively stable over a long period of time and although suspensions less than 6 months old are ordinarily employed, the comparative results obtained with a suspension 14 months old emphasize the stability of these preparations.

DISCUSSION

The results described herein suggest that the collodion agglutination test is not a complex procedure. In our experience about ten days are required for the preparation of the particles but since approximately three days are concerned with the drying of the particles in vacuo and about four days are spent bubbling off the acetone by aeration, a negligible amount of observation is required in these processes. The first step of precipitating the collodion in water with subsequent washing of the mass obtained requires about an hour of

the first day. After the drying procedure, a full day is spent obtaining the particles by repeated precipitation with water-acetone mixtures and redissolving with acetone as described by Cavelti.[9] Following the bubbling off of the acetone, the final day is spent in washing the particles. Thus actually two full days and an hour of a third day require close attention. The subsequent stability of the particles and the simplicity of performing the test which merely requires the sensitization of the collodion by histoplasmin for an hour during which time the serum dilutions are made is obvious. After admixture of the sera and antigen, the short two-hour incubation period makes it possible for tests to be set up in the morning and read in the early afternoon. This technique appears to be less time consuming and cumbersome than the hemagglutination test. In the latter it is necessary to prepare washed cell suspensions frequently prior to sensitization of them and then one must be concerned with the adsorption of normal hemagglutinins from each serum specimen tested. In addition the 1:2 optimal dilution of histoplasmin described in Norden's report[7] denotes the requirement of a far greater amount of antigen than does the collodion agglutination test where hundredfold dilutions of histoplasmin are usually sufficient to sensitize the particles.[2] Thus, in our experience the collodion agglutination test has offered a greater economy of time and materials.

From the foregoing it is not to be construed that the collodion agglutination technique may be applied to any antigen. The important factor in its use is to first obtain a clear soluble antigen relatively free of impurities. Although histoplasmin adapts itself readily in this procedure, it may ultimately be replaced by a more purified chemically defined fraction which would tend to be more specific. Landy's studies[11] in the Department of Biologic Products at this institution have demonstrated marked reactivity and specificity by adsorbing purified Vi antigen preparations onto collodion. His observations using both our old and new lots of collodion and his own preparation have shown comparable specific results in the presence of specific immune sera. On the basis of the similar results obtained by both laboratories in this institution, it is felt that evidence has been presented that collodion preparations are relatively stable and apply themselves adequately to certain antigens. The quality of the test obviously is dependent upon the concentration and relative purity of the antigen which must offer a clear solution when dissolved.

A more rapid method for the preparation of collodion particles described by Havens and Lloyd[8] affords material which is said to be stable for seven to fourteen days. As demonstrated our preparations are stable over a much longer period. One would, then, have to evaluate his needs as to which method of preparation would be most desirable.

The supply of collodion in this laboratory usually requires replenishment in five to six months and it has rarely been necessary to use older lots. Although the comparative studies shown herein do indicate the relative stability over a longer period, it is not meant to imply that the very old batches are used in routine studies. Spot checks of multiple samples inserted into our routine testing program at periodic intervals have demonstrated a high degree of reproducibility of results.

CONCLUSIONS

Further experiences with the collodion agglutination test for histoplasmosis are discussed. The relative stability of the collodion particles, the simplicity and repeatability of the test, and the economy of materials have been demonstrated. A comparison of three different lots of collodion varying in age from 3 weeks to 14 months yielded similar results in the presence of positive and negative human and rabbit sera.

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AN IMPROVED SKIN ELECTRODE

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SEVERAL types of electrodes, suitable for use in the measurement of skin resistance or other bioelectric phenomena, have been tested in this laboratory during the past four years. The electrode shown in Fig. 1 has the following attributes: it is quickly and easily applied, exerts a fairly constant pressure, stays in place during minor movements of the extremities, has a neat appearance, is comfortable to the patient, can be cleaned easily, and, further, can be adapted easily for use over a wide age range of subjects. The electrode is constructed of Plexiglas and is rugged enough to withstand prolonged use.

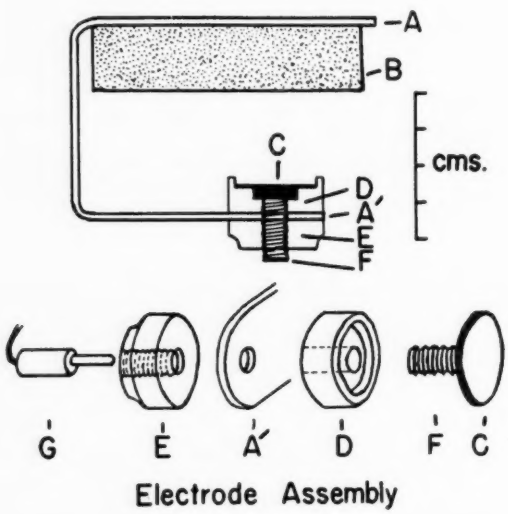

Fig. 1.

It consists of a band of Plexiglas ¾ in. wide and ⅛ in. thick, *A* to *A'*, bent, by *carefully* warming in a soft flame, to the shape shown. The foam rubber pad, *B*, which is cemented to the Plexiglas can be cleaned easily with soap and water or replaced when necessary. The electrode assembly consists of a metal plate, *C*, and a threaded metal rod, *F*, which holds the two Plexiglas units, *E* and *D* (readily turned on a lathe from rod), onto the strip. The threaded member, *F*, is drilled so as to accept the plug, *G*. The metal used for the plate,

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C, will depend, of course, upon the use to which the electrode is put. The pressure exerted by these electrodes on the palmar surfaces depends on the distance between the surfaces *B* and *C*, and this distance may be readily varied. If this distance is about one-half the thickness of the palm, a firm but comfortable pressure is exerted.

Although we have used these electrodes mainly for the measurement of galvanic skin resistance, they would find other uses where similar electrodes are needed, as in electrocardiography and the measurement of skin impedence.

A USEFUL MENSTRUUM FOR DRYING ORGANISMS AND VIRUSES

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DESICCATION of bacteria from the frozen state for the preservation of virulence and vitality has proved its usefulness in stock culture collections. Vaccines containing living organisms or viruses, such as brucellosis and yellow fever prophylactics, are more conveniently controlled and distributed if in the dried state. The menstruum herein described should be useful in desiccation of organisms because it is nonantigenic and more efficient than many other menstruums.

Many vehicles have been used for suspending cultures prior to freezing and drying. Naylor and Smith[1] reported that a mixture of ascorbic acid, thiourea, ammonium chloride, and dextrin was a good suspending medium for freezing and drying *Serratia marcescens*. They did not compare this mixture with milk, which Anderson[2] reported to be the best menstruum for suspending rickettsiae. Anderson found that next to milk, in order, were yolk sac, Tyrode's solution, guinea pig serum, broth, and water. He measured survival in these vehicles on standing. He did not freeze or dry. Ficker[3] found that the best menstruum for drying was milk; next in order were serum, bouillon, saliva, water, saline, and urine. Topping[4] found milk the best suspending medium for preservation of rickettsiae by drying. Leifson[5] reported greater viability when organisms were dried in broth and blood than when dried from water. Frobisher and co-workers[6] recommended serum as a vehicle. Delpy and Chamsy[7] used fresh horse serum in drying diphtheria cultures. Dried from saline, the survival time was less than when serum was used. Weiser and Hennum[8] reported that inactivated rabbit serum or skim milk was superior to 1 per cent peptone as a vehicle for drying *Escherichia coli*. Verge and Goret[9] found that 10 per cent gum acacia added to serum was a more suitable menstruum than serum for drying distemper virus. Stamp[10] used a menstruum of peptone, sodium chloride, beef extract, gelatin, and ascorbic acid. Curasson and Philippe[11] dried plague organisms in 1 part ground spleen and 5 parts buffer. Tanner and Wallace[12] found that organisms frozen at a pH of 4.0 to 5.5 suffered a considerable mortality as compared with those frozen in an alkaline medium. (Increasing the sodium chloride content decreased the viability.) Increasing the amount of sucrose, however, increased the viability. Rosenthal[13] found lactose a good menstruum for desiccation of BCG vaccine. Hampp[14] used a mixture of hormone broth and gastric mucin in drying spirochetes.

It will be noted from the foregoing that milk ranks at the top as far as efficiency is concerned, when comparable tests are made. Milk has been used as a vehicle for drying organisms in the Laboratory of Biologics Control since 1941.

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Many of the aforementioned vehicles have disadvantages. Milk and some of the others mentioned are antigenic and would be unsuitable for injection into human beings. Human serum may contain the agent or agents of viral hepatitis and possibly other viruses. Serum and blood, especially when fresh, may kill certain organisms. Organisms which coagulate milk, such as *S. marcescens,* will tend to be clumped by the coagulum, making bacterial counts difficult. Broth is difficult to dry from the frozen state. It becomes concentrated during the drying process, tends to melt and to be drawn up into the drying apparatus.

METHODS AND RESULTS

In searching for a suitable drying menstruum, a milk dialysate was found to be effective. This was prepared by suspending cellophane sausage casing containing 100 c.c. of normal saline in a quart of milk. After standing overnight in the cold room the casing was washed. The contents were then removed and used as the suspending medium. Cholera organisms were used in testing this menstruum since they are notoriously difficult to dry without great loss of viability. Cholera No. 6, an avirulent but smooth culture, grown overnight on horse meat infusion agar at 35° C., was suspended in water and a 1:10 dilution of this was made in the milk dialysate, in milk, and in dialyzed milk. Counts were made of the three suspensions by the usual plating methods. Two-tenths milliliter amounts of the suspensions were placed in shell vials and frozen at −18 to −20° C. Several vials were dried in the cold room at 5° C. over 95 per cent sulfuric acid under a vacuum. They were opened after several days of storage and the organisms counted. Bacterial counts were also made on several of the frozen ampules. The results are recorded in Table I.

TABLE I. BACTERIAL COUNTS (IN MILLIONS) OF CHOLERA ORGANISMS FROZEN AND DRIED IN VARIOUS MENSTRUA

	BEFORE FREEZING	AFTER FREEZING	FROZEN 3 DAYS	WHEN DRIED 3 DAYS	WHEN DRIED 10 DAYS
Milk	148	72	112	24	7.9
Dialyzed milk	220	20	18	5	0.5
Milk dialysate	118	22	15	5	2.3

There is evidently considerable loss in viable count when cholera organisms are frozen and a further loss when dried. The milk dialysate appears to be as good or better than the dialyzed milk. In view of this, a solution was prepared which would contain the dialyzable components of milk. This mixture had the following composition: potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$) 1.35 Gm., sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) 2.45 Gm., potassium phosphate (K_2HPO_4) 0.61 Gm., calcium chloride ($CaCl_2$ anhydrous) 1.33 Gm., magnesium chloride ($MgCl_2 \cdot 6H_2O$) 0.6 Gm., potassium carbonate ($K_2CO_3 \cdot 1\frac{1}{2}H_2O$) 1.0 Gm., lactose 57.5 Gm., lactic acid q.s. to pH 7.00 (about 0.65 c.c.), H_2O 1,000 c.c. Dissolve all ingredients except $CaCl_2$ in 500 c.c. water. Dissolve $CaCl_2$ in 500 c.c. water. Mix the two solutions and adjust pH. Filter through a Berkfeld filter.

To compare the efficiency of this lactose salts mixture with milk, various organisms were suspended in the mixture and dried. Comparable preparations of the same organisms, suspended in milk and dried at the same time, were used as controls. The culture suspensions were diluted 1:10 in the test vehicles and dried by means of a sulfuric acid desiccator[15] using 95 per cent sulfuric acid. The percentage of viable organisms was determined by plate counts before and after drying and appears in Table II.

TABLE II. PER CENT SURVIVAL OF VARIOUS ORGANISMS DRIED IN MILK AND IN LACTOSE-SALTS MIXTURE

ORGANISM	PER CENT SURVIVAL	
	IN MILK	IN LACTOSE-SALTS MIXTURE
Neisseria catarrhalis	1.2	6.3
Neisseria catarrhalis	90	180*
Neisseria catarrhalis	5.2	21
Vibrio comma	0.004	0.003
Vibrio comma	0.9	7.2
Vibrio comma	1.6	7
Serratia marcescens	76	60
Serratia marcescens	9	21
Serratia marcescens	7	35
Brucella abortus (19)	41	72
Brucella abortus (19)	0.8	3.0
Brucella abortus (19)	4	51
Escherichia coli	43	75
Escherichia coli	14	30

*Probably due to disaggregation of clumps.

From inspection of Table II it is clear, with the organisms tested, that this vehicle shows considerable superiority over milk which has heretofore been considered by many as the best material for this purpose.

Serum, recommended by some, has been found inferior to the lactose-salt mixture when tested with *Esch. coli.* Thirty-four per cent of the organisms survived drying in lactose-salt mixture while 11 per cent survived in horse serum. After storage of the dried cultures for six days in the cold in a vacuum, the percentages of survival were 14 and 2 respectively.

One will note in Table II that the per cent of surviving organisms varied from test to test even though the same strain was used. It must be remembered that in each experiment the suspensions of bacteria were treated exactly alike and were dried on the same apparatus at the same time. However, the fifteen experiments were conducted on different days and differed in certain particulars such as age of culture, dryness of media, time between preparing suspensions and drying, time between desiccation and counting, and temperature during drying and storage. Some of these variables are important. For instance, if the whole drying process takes place at 5° C., a larger percentage of organisms is viable than if drying is done at 35° C. Proom[16] has shown that younger cultures survive better than older ones. These factors may account for many of the discrepancies in Table II.

In order to compare other vehicles with this material, cholera organisms suspended in Naylor's medium, milk, 5 per cent lactose, 10 per cent lactose, 15 per cent lactose, broth, and the described mixture were dried and studied for

viability. The sulfuric acid manifold apparatus[15] was used for drying with 95 per cent sulfuric acid. The results are expressed in Table III.

It is clear that the lactose-salt mixture is superior to the other vehicles tested as far as the cholera organism is concerned. Evidently lactose alone is not responsible for the good results. On the other hand, a solution containing only the salts present in the menstruum, without the lactose, was found to

TABLE III. PER CENT SURVIVAL OF V. COMMA DRIED IN VARIOUS SUBSTRATES

Lactose-salt mixture	7.2
5% lactose	0.05
10% lactose	0.02
15% lactose	0.003
Naylor's solution	0.0000
Milk	0.9
Broth	0.21

be inferior to the whole mixture. It is quite possible that several of the salts present in the mixture are unnecessary. We have not tested to see which, if any, are superfluous.

In our experience cysteine failed to improve the salt-lactose menstruum. Without cysteine we obtained 0.6 per cent survival while with 0.1 per cent neutralized cysteine HCl we obtained 0.1 per cent survival.

It is difficult to account for the poor showing made by Naylor's medium. It is possible that the thiourea used here was not pure and was toxic to the organisms.

To see if the lactose-salt vehicle would be suitable for drying viruses, dried yellow fever vaccine was reconstituted in water. It was suspended 1:10 in saline, in human serum, and in the lactose-salt mixture. It was then dried from the frozen state over sulfuric acid. The original suspension and the three dried products, after reconstitution, were titrated by serial intracerebral injection into mice. The results are expressed in Table IV.

TABLE IV. POTENCY OF YELLOW FEVER VACCINE BEFORE AND AFTER DRYING IN SALINE, 10 PER CENT HUMAN SERUM, AND THE LACTOSE-SALTS MENSTRUUM

	LD_{50} DILUTION WHEN INJECTED INTRACEREBRALLY IN MICE			
		DRIED IN		
DATE OF TEST	VACCINE BEFORE DRYING	N. S.	HUMAN SERUM	LACTOSE-SALTS MENSTRUUM
8/26/49	1/1,700	1/320	1/190	1/10,000
9/19/49	1/395	1/10,000	1/3,150	1/3,900
9/20/49	1/10,000	1/10	1/10	1/5,500
10/14/49	1/32,000	1/39	1/2,000	1/3,900
Average	1/11,024	1/2,592	1/1,337	1/5,825

It appears that this mixture is suitable as a menstruum for the desiccation of yellow fever vaccine and possibly for other viruses.

Yellow fever vaccine as shown in Table V is relatively stable when incubated in this menstruum.

TABLE V. LOSS OF POTENCY OF YELLOW FEVER VACCINE ON INCUBATION IN SALINE, 10 PER CENT HUMAN SERUM, AND THE LACTOSE-SALTS MENSTRUUM

	LD_{50} DILUTION WHEN INJECTED INTRACEREBRALLY IN MICE					
		VACCINE SUSPENDED IN				
DATE OF TEST	VACCINE BEFORE INCUBATION	N. S.	HUMAN SERUM	LACTOSE-SALT MIXTURE	TIME OF INCUBATION	TEMP. OF INC.
9/14/49	1/10,000	1/1,000	1/32	1/1,000	7 hr.	37° C.
10/14/49	1/32,000	1/115	1/13	1/1,000	3 hr.	42° C.

One disadvantage of this menstruum is the fine fluffy nature of the dried product. When an ampule is opened, especially if it has been sealed in a vacuum, the contents may tend to fly about. If the material dried has a rather high protein content such as yellow fever vaccine, this does not occur. It is possible that in some cases a colloid such as acacia might be added with advantage to the menstruum. This will cause the material to dry in a more cohesive mass.

SUMMARY

A suspending menstruum for drying bacteria and viruses has been described. It is efficient in preserving viability, readily available, nonantigenic and contains only lactose, citrates, and inorganic salts.

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A SIMPLE METHOD FOR THE DENSIOMETRIC DETERMINATION OF HEAVY WATER

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ALTHOUGH the falling drop method for the determination of deuterium oxide yields excellent results, equipment is required which is not always readily available. In the present communication a method is described for the determination of the density of deuterium oxide-water mixtures with an accuracy of 3 parts in a million with equipment no more complicated than a Beckman differential thermometer.

In this method, advantage is taken of the fact that the specific gravity of an aqueous solution of deuterium oxide decreases more rapidly with temperature than does the specific gravity of a hollow Pyrex glass bead. If the temperature at which such a bead is in equilibrium with the solution is noted and compared with the temperature required for equilibration of a set of known standards, the deuterium oxide content of the unknown is readily determined.

A glass bead is prepared from 5 mm. Pyrex tubing and its specific gravity adjusted by addition or removal of a fine glass rod until the bead just sinks in redistilled water at 20 to 25° C. Such a bead can be prepared by trial and error in 1 to 2 hours. The volume of the bead we have used routinely is about 0.5 $cm.^3$ but beads as small as .05 $cm.^3$ have been prepared and used.

The bead is placed in a small Pyrex test tube (O.D. 10 mm.) and covered with redistilled water. The water is boiled with the bead to remove dissolved air and the tube is stoppered. The tube is then clamped in a vertical position in a 2 liter beaker equipped with a mechanical stirrer and the bulb of a Beckman thermometer is placed next to the tube.

If the bead floats at the temperature of the water, a Bunsen flame is played on the side of the beaker momentarily or until a temperature is reached at which the bead sinks. Since temperature equilibration is not immediate, no attempt is made to read the temperature at this point.

The beaker and its contents are permitted to cool in air and the bead is observed closely. The temperature at which the bead just breaks away from the bottom of the tube is noted to the hundredth of a degree and considered the end point. The determination may be repeated as often as required.

This process is then repeated with solutions containing amounts of deuterium oxide varying from 0 to 2 per cent. The results of such a calibration are given in the accompanying figure.

The temperature required to float the bead in the unknown solution is then determined and the deuterium oxide concentration read from the chart. One calibration of the bead has been found to be sufficient, as no changes are observed from day to day.

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The end points are duplicable to .01° C. in the hands of students who are unfamiliar with the apparatus. With our beads we have found that a deuterium oxide concentration increase of 1 part in a thousand changes the end point temperature by 0.35° C. Consequently this method will distinguish between deuterium oxide concentrations of 3×10^{-5}, and a 1 per cent deuterium oxide solution can be determined with an accuracy of 0.3 per cent.

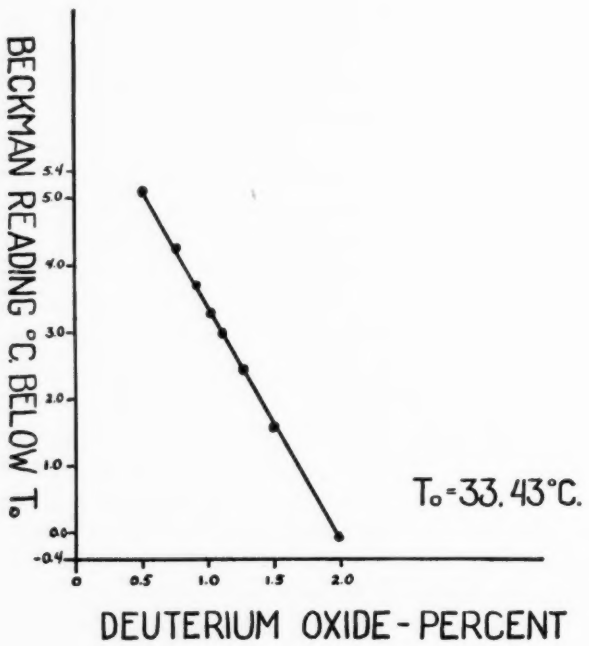

Fig. 1.

When a Beckman thermometer is not available, readings of sufficient accuracy for work with 1 per cent heavy water can be obtained with a chemical thermometer graduated in tenths. The error in the determination of 1 per cent deuterium oxide with such a thermometer is 3 per cent if the reading is made to the tenth of a degree.

When the large bead (0.5 cm.^{3}) is employed, quantities of sample of 2 ml. are required. Use of the smaller (.05 cm.^{3}) bead permits determinations on 0.2 ml. samples with the same accuracy. Still smaller beads require even less sample, but we have not found it necessary to employ these.

THE USE OF AMPIN, AN AUTOMATIC AMPULE INJECTOR FOR ROUTINE AND EMERGENCY HYPODERMIC MEDICATIONS

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SINCE January, 1949, the Committee on Drugs and Formulary of Bellevue Hospital of New York has had under consideration the use of an automatic hypodermic device to facilitate subcutaneous and intramuscular injections of the commonly employed parenteral medications. A study of the practicability and safety of this device as employed by the usual hospital nurses, attendants, and resident staff was set up under the supervision of a subcommittee. The purpose of the investigation was to determine (1) whether the device offered any advantage over the ordinary syringe-needle method of administering hypodermic medications and (2) the practicability and ease of use of the automatic ampule under routine and emergency conditions as required in large municipal hospitals.

METHODS AND APPARATUS

Description of Ampin.—The automatic ampule injector known as Ampin is supplied as a disposable unit, sterile and ready for instant use. It consists of an ampule which contains the medication under pressure of an inert gas, helium; a flexible connector tube; a filter; a needle attached to the connector tube by means of a hub; and a needle cover. These are depicted in Fig. 1. From information obtained from the manufacturer the following facts pertaining to the various parts of the Ampin are pertinent. The glass ampule is made of a resistant glass, Neutraglass, with a specially constructed tip allowing for easy breakage when the Ampin is to be used. The entire ampule, including the tip, is sufficiently strong to withstand normal handling as well as reduced pressure equivalent to a vacuum of 26 inches. Furthermore, the Ampin can withstand manipulation such as simulated pressure altitude of 100,000 feet at mean temperature of 80° F. and −60° F., and explosive decompression from 8,000 feet to 35,000 feet in 2½ seconds. The rubber connecting tube is transparent and fungus resistant. The latter quality is achieved by impregnating a fungicide within the outer layers of the rubber during the fabricating process. The filter used for clear solutions is made of a lint-free balloon cloth of low absorption value. In the Ampins containing suspended materials such as penicillin in oil, the filter is an alloy wire screen. The stainless steel needle for the Ampin to be used subcutaneously is of 22 gauge and is ½ inch long, while that for intramuscular use is of 20 gauge and is 1½ inches long. The needle hub consists of nickel plated brass. The sterility of the needle is protected by a heavy glass cover capable of withstanding a crush resistance of 60 pounds. The cover is sealed to the lower end of the rubber connecting tube by means of ethocel, a plastic permitting a water-insoluble seal which is clear and tack-free on drying. The whole device is either sterilized as a unit after assembly of the various parts or, as in the case of penicillin or other heat labile medications, it is assembled aseptically following appropriate sterilization of the solution.

A report by a subcommittee of the Committee on Drugs and Formulary, Bellevue Hospital.

The authors constitute the subcommittee of the Committee on Drugs and Formulary of Bellevue Hospital of New York. Other members of the Committee are Dr. Arthur C. DeGraff (Chairman), Dr. Frank C. Combes, Dr. Anthony M. Siragusa, Dr. J. James Smith, Dr. Max Trubek, and Dr. S. Bernard Wortis. The Committee also included Mr. Ludwig Zwilling, senior pharmacist of Bellevue Hospital, and the consultants . Dr. Robert A. Lehman, pharmacoloist, Department of Therapeutics, New York University-Bellevue Medical Center, and Mr. Meyer Walitsky, of the Office of Controller, City of New York.

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Use of Ampins for Routine and Emergency Purposes.—To determine the efficacy and safety of the Ampins as they would be used under routine and emergency conditions, the Ampins were made available to four special hospital services. These included the emergency ward, the dressing office and emergency first-aid room, the ambulance service, and the operating rooms. The latter will be considered later since the use of hypodermic medication during operative procedures requires special techniques. In all services, whenever a drug available in Ampin form was to be administered, it was used routinely in place of the

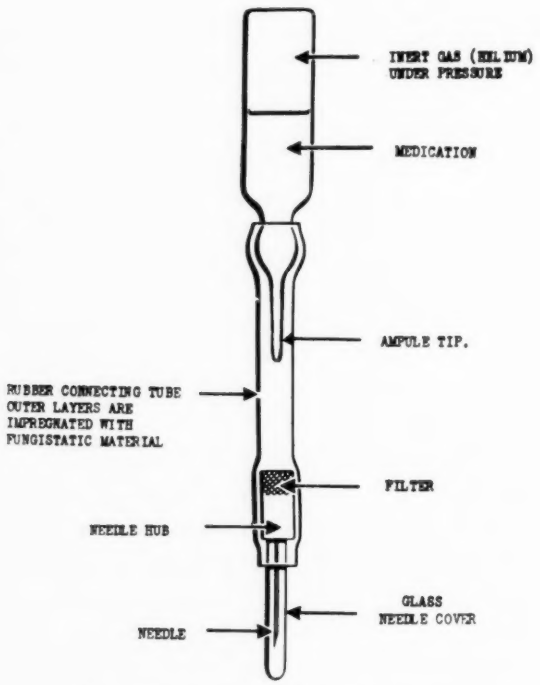

Fig. 1.—Schematic diagram of Ampin.

previous practice of utilizing the syringe-needle method and obtaining the drug in the form of ampule, vial, or hypodermic tablet. The drugs available in Ampin form which were utilized in this investigation were morphine sulfate in 10 and 15 mg. doses, Demerol hydrochloride 100 mg., epinephrine hydrochloride 1:1,000 in 0.5 and 1 c.c. doses, ephedrine sulfate 50 mg., caffeine sodium benzoate 0.5 Gm., nikethamide 1.5 c.c. of a 25 per cent solution, and crystalline procaine penicillin G with aluminum monostearate, 1 c.c. containing 300,000 units. With the exception of penicillin Ampins which were given intramuscularly, the other Ampins were for subcutaneous administration.

The technique of using the Ampin was demonstrated once to the members of the staffs of each service. Thereafter, over a six-month period, the use of the Ampins was observed carefully to determine the practicability, ease of use, and safety. The use of the Ampin was found to be very easy to learn by all staff members and student nurses. Usually after two or three trials one becomes proficient in its application. The technique is simple and requires only a few precautions. It consists essentially of the following steps. (1) The skin site is prepared with an antiseptic in the accustomed manner. (2) The Ampin should be inspected to avoid the occasional broken one. (3) The needle cover is removed. (4) Holding the Ampin by the needle hub, the needle is inserted into the selected site. For subcutaneous use the angle of insertion should be 45 to 90 degrees. For intramuscular injection the angle should be 90 degrees. (5) The rubber flexible tubing immediately above the needle

hub is pinched and released. If the needle has penetrated a vein, this will be reflected by blood entering the tubing. This is clearly visible through the transparent rubber. (6) The ampule tip is broken by bending up the flexible connecting tube. (7) The Ampin is held in a "bottoms up" position so that the inert gas under pressure is above the solution to be injected. Failure to do so results in the gas escaping without forcing the solution into the injection site. (8) The Ampin is held in an upright position until the entire solution is cleared through the filter before removal of the needle from the injection site.

Approximately 1,700 Ampins containing the previously listed drugs were used under direct supervision in the emergency ward, dressing office, and ambulance service. Many more Ampins were used for demonstration and teaching purposes but were not tabulated because they were not used for routine purposes.

It was at once apparent that the Ampins possessed many advantages over the accustomed syringe-needle hypodermic methods. The drug was always available in a sterile form for instantaneous use. This was of tremendous help to ambulance surgeons who were able to give an injection in a matter of seconds. Previously an injection required assembly of the syringe and needle which were kept in a sterile pack or in alcohol containers and the removal of the required medication from ampule or vial. The same advantage was appreciated in the other emergency services. The economy of this can be only roughly estimated since when a hypodermic medication is administered one thinks only of a sterile syringe and needle. Little thought is given to the time and labor required for the nurse to wash, clean, and sterilize these syringes and needles. Furthermore, the accessory equipment required such as sterile forceps, sterile containers for the forceps, syringes and needles, antiseptic solutions or alcohol, sterilizing equipment varying from water and steam apparatus to autoclaves are never considered.

By the syringe-needle technique, excluding the actual time required for sterilization of the equipment, additional time is required to fill the syringe with the medication. Since this is available in three forms, ampule, multiple vial, or hypodermic tablets, three different techniques must be mastered. Sterile precautions must be used throughout with each step. This is subject to many pitfalls, making contamination a definite possibility. The actual time required to fill a syringe, assuming that the syringe and needle were available in sterile form, was tested by accurately timing a group of 138 nurses under conditions of emergency use of hypodermic medications. If the medication was available in the form of a tablet requiring solution by boiling in sterile water or saline, the time required to have it ready for administration varied between 1 min. 22 sec. and 4 min. 8 sec., with an average of 2 min. 46 seconds. The time required to break an ampule and fill the syringe varied between 23 sec. and 1 min. 55 sec., with an average of 55 seconds. The shortest time was by ultilizing a multiple dose vial, which required 20 sec. to 1 min. 36 sec., with an average of 41 seconds. Thus, varying with the experience of the nurse and the type of hypodermic medication to be used, it required on the average from 41 sec. to 2 min. 46 sec. before the patient would be given the medication. For emergency conditions this is not satisfactory. Under such circumstances it was at once apparent that since no time is consumed in preparation of the Ampin, the Ampin administration of emergency drugs is the method of choice. The disposable sterile unit obviated the necessity for time-consuming preparation of sterile syringes and needles. It obviated the economic loss of broken syringes and continuous replacement of sharp needles.

Although penicillin is not considered an emergency measure, the use of this medication in Ampin form lent itself very satisfactorily for use in emergency services. In a busy accident ward, the time required for the nurses to administer penicillin by the syringe-needle technique is considerable. This was definitely eliminated by the use of the Ampin and the nurse was freed for more important duties.

Use of Ampin in Operating Room.—In the operating room the Ampin has been of considerable value since it provides a convenience and accuracy of dose as well as an economy of time and service. The use of approximately 300 Ampins was observed. Ephedrine had been used most often, in the majority of patients with spinal anesthesia. Epinephrine had been used frequently and caffeine sodium benzoate occasionally. The use of morphine and Demerol in Ampin form was particularly satisfactory.

The use of the Ampin has eliminated many objections inherent in the use of the syringe-needle technique of administering hypodermic medication in the operating room. It has been the custom whenever the anesthetist requires a particular medication to either prepare it himself or request a nurse who has many duties to perform hurriedly and often in an atmosphere of considerable nervous tension to do so. To prepare the hypodermic himself, the anesthetist must by necessity be less attentive to the patient's anesthetic needs. It requires the use of ampules and vials of particular medications which must be readily available. Ampule breaking devices in a sterile form must be kept close at hand. Packets of sterile syringes and needles, previously prepared by nurses, must be easily available. The manipulations required to prepare and give the hypodermic injection provide many chances for violating aseptic techniques. For the nurse to obtain the hypodermic medication necessitates an additional nurse not directly assisting with the operation who is always available for such an errand. It usually requires a hurried dash to a cabinet immediately outside the operating room, the procurement of the medication, the various procedures necessary to transfer the medication to a sterile syringe, and then the wrapping of the syringe with needle attached in a small piece of gauze and the placing of it at the anesthesia table which is rarely clean. This particular bad practice and the shortcomings of syringe-needle technique have been eliminated by the use of Ampins which are directly under the domain and responsibility of the anesthetist. This, in addition to the obvious advantage of reducing the hazards of violating aseptic technics, has been of immense value in the proper management of the patient. The convenience of the Ampin for the anesthetist working alone is considerable. He is able, if it is desired, to manipulate the injection easily with one hand while the other is occupied with holding the patient's head in a certain position or any of the other exercises that he may not want to discontinue even temporarily. He need not concern himself with the used syringe and needle which under conditions of stress are often broken.

The accurate dose that is assured with the Ampin is of significance to the anesthetist. The chance of losing some of the solution by the syringe-needle technique is considerable. In checking this possibility without the nurse's knowledge, it was determined in thirty observations that the exact amount ordered was given less than one-half the times attempted.

Depth of Distribution of Solution at Site of Injection.—Since the solution in Ampins is ejected by means of an inert gas, helium, under approximately two atmospheres of pressure, several questions must be considered. First, to what distance from the needle opening will the solution be ejected? To determine this factor, specially prepared Ampins containing 1 c.c. of a 10 per cent solution of sodium iodide were used. The radiopaque nature of this drug allowed accurate x-ray evaluation of the depth of penetration when the Ampin was activated. Ten subjects with varying thicknesses of adipose tissue were chosen. The subcutaneous type of Ampin with a ½ inch, 22 gauge needle was used throughout. The needle of the Ampin was inserted to the hub at varying angles from 3 to 90 degrees. The x-ray was taken with the plane of the Ampin parallel to the film. A control film was always taken to indicate the depth of needle insertion. The solution was injected and another film taken immediately thereafter. Fig. 2 illustrates an actual experiment and the distribution of the solution at the site of injection. The spread from the needle opening never exceeded 21 mm. and was usually 7 to 12 mm., averaging 8.4 millimeters. The solution was evenly distributed regardless of angle of injection or whether the site of injection was intramuscular or subcutaneous. Thus, the solution was found to be distributed to an equidistance in the direction opposite to the needle opening as well as to the sides. The only exception influencing the distance of penetration of the solution was the tissue in close proximity to the direct line of the needle opening. If the needle opening was near muscle or fascial layers, the solution would be distributed up to the line of demarcation of subcutaneous tissue and muscle and present a more lateral spread. It is of interest to note that the ½ inch needle when inserted at 90 degrees may have its opening in muscle rather than subcutaneous tissue. This is particularly so if the individual is thin.

Safety of Injection.—The second question of importance is the possibility of injecting gas with the Ampin. For determining this factor, an experiment similar to the former one but using sterile saline was performed. Gas entering the subcutaneous tissue is readily

visible by x-ray technique. It is possible to study not only the degree of distribution but also the length of time required for absorption. Twelve subjects were used. The presence of gas was easily detected at the site of injection in all instances. As in the case of the solution there was a tendency of equal distribution in all directions, but differed from the former in that a greater spread occurred along tissue planes. The distance from the needle opening varied between 8 and 44 mm., with an average of 20 millimeters. The amount of detectable gas was in each instance less than would be expected if all the gas of the Ampin entered the tissue.

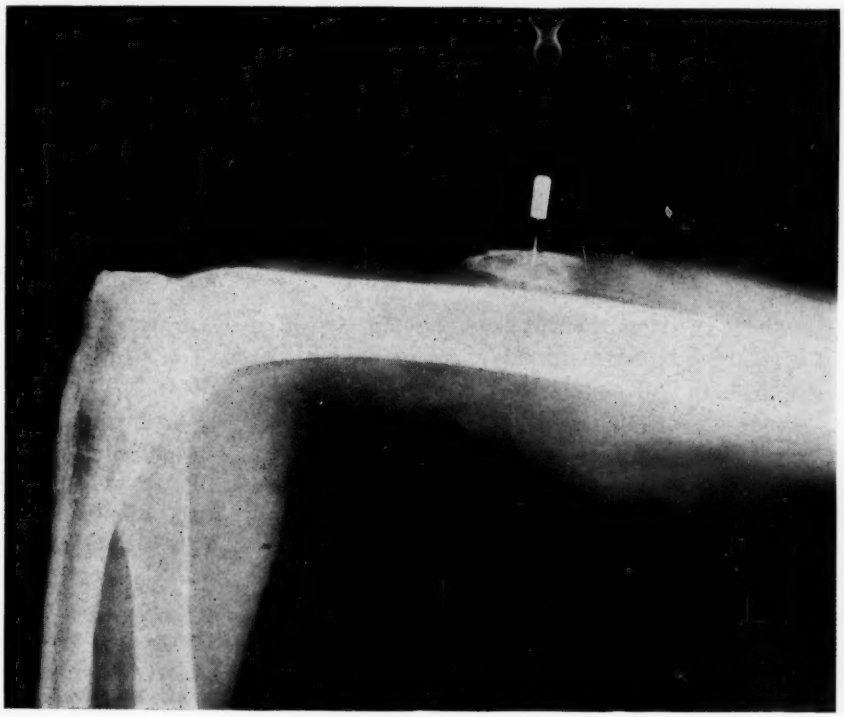

Fig. 2.—Distribution of solution (10 per cent sodium iodide) in deltoid region upon activation of Ampin.

The absorption of the inert gas from the injection site was slow. Although in the majority of instances it would no longer be detected at the end of three hours, in a few instances the gas was still visible on x-ray films taken twenty-four hours after the injection. The presence of gas in the tissues therefore requires a precautionary note if diagnostic x-rays are contemplated in the vicinity of the injection site. It should not be confused with gas bacillus infections or spontaneous subcutaneous emphysema.

Since the inert gas enters the injection site, the question arises as to the possibility of gas embolism. With ordinary precautions usually taken with the Ampin to forestall intravenous injection, this possibility is extremely remote. Furthermore, since under normal operation the maximum amount of gas that could be injected is less that 1.5 c.c., the dangers of a fatal air embolism would be very unlikely. Even if the Ampin should be improperly used, i.e., "bottoms down" position in which none of the medication is injected, the maximum amount of gas that could be injected is 2.6 cubic centimeters. Much larger quantities of air have been inadvertently and harmlessly injected during the course of transfusions and infusions. As much as 5 to 10 c.c. of air have been injected rapidly in man without producing

clinical symptoms.[1, 2] Moore and Braselton[3] on the basis of animal experiments, have estimated that as much as 37.5 c.c. of air can be injected into a pulmonary vein of man without serious consequences. In view of these facts the likelihood of air embolism is very remote.

The third factor for consideration is the possibility of glass from the broken ampule entering the site of injection. The filters are effective barriers to the fragments of glass. Since the glass is radiopaque, this was clearly seen in the x-ray studies. In no instance was any glass discernible in tissue when the solution was injected. By direct chemical analysis, however, approximately 13 gammas of glass per Ampin are ejected through the needle. This is considerably less glass than is injected by the syringe-needle technique where the ampule is filled, broken, and the solution drawn up into the syringe.[4]

Pain and Tissue Damage at Site of Injection.—In view of the fact that the solution is ejected under pressure, it may be asked whether such an injection is more painful and whether the gas that enters the injection site results in undue irritation. The degree of pain experienced by the patient is in no way different from that noted with the ordinary syringe-needle method. There is no tearing of tissues but rather a gradual separation incident to the volume of injected solution. The gas, since it is inert, does not initiate any pathologic changes and is eventually absorbed.

DISCUSSION

Advances in drug therapy and particularly in new drugs for parenteral use have by far exceeded any advances in their methods of administration. It is of interest to note that the ninth revision (1905) of the *United States Pharmacopoiea* listed 1,039 drugs of which 20 or 1.8 per cent could be used parenterally. Only 4 or 0.3 per cent were for sole use by hypodermic measures. Contrast this to the thirteenth (1947) revision where 92 or 17 per cent of the 656 items listed were for parenteral administration. Of these 70 or 10.7 per cent were for parenteral use only. A similar story is available from analysis of the *New and Nonofficial Remedies* yearbook published by the American Medical Association. In 1911, excluding serums and vaccines, there were 303 drug items listed of which 26 or 8.6 per cent could be used parenterally. Of these, 9 or 2.9 per cent were for exclusive hypodermic administration. In 1949, 127 drugs (38 per cent), exclusive of serums and vaccines, of 335 items listed were recommended for hypodermic use, while 66 or 19 per cent were for exclusive parenteral use.

It is apparent that the medical profession is becoming more reliant upon hypodermic medications. Not only are new and effective drugs being introduced that rely on hypodermic techniques, but also new and more effective uses are being found for old drugs. It is, therefore, no wonder that techniques and apparatus for injections of drugs should attempt to keep pace with advances in drug therapy. Until the introduction of the Ampin, improvements in hypodermic methods have been few and resolved principally about syringes and needles. One need only mention the many advantages in the use of Ampins that have become evident during the course of this investigation to realize that the Ampin has satisfactorily answered many of the objections inherent in the familiar syringe-needle method of hypodermic medication.

1. A safe, reliable, and easy method is available for the routine and emergency administration of commonly used parenteral medications.

2. The drug is always available in a sterile form for instantaneous use. Time-consuming preparation of hypodermic solutions and filling of syringes by a variety of techniques are eliminated.

3. The economy in time, service, and actual cost of equipment including syringes, needles, sterilizing apparatus, and so on, is a major factor offsetting the higher cost of the Ampins.

4. The Ampin is of great value in the operating room where more efficient control of aseptic technique is possible. They are of great assistance to the anesthetist for better control of the patient and eliminate many undesirable features which are inherent in the use of syringes and needles.

5. The possibility of syringe-transmitted hepatitis is eliminated. The virus for this infection, being heat resistant, is not killed by the ordinary methods of sterilization. The dangers of syringe-needle technique as an important factor for transmission have been emphasized adequately in an editorial of the *Journal* of the American Medical Association.[5] The Ampin appears to be the logical answer to avoid this condition.

6. The physician is assured an accuracy of dosage which is not dependent upon experience and technique of the individual administering the medication.

SUMMARY AND CONCLUSION

The practicability, ease of use, and safety of Ampin, a new automatic hypodermic device, were investigated over a six-month period. It was found to be a safe and practical method for the routine and emergency use of hypodermic medications. It was of particular value in the emergency first-aid room, ambulance service, and operating rooms. Its use not only eliminated many of the objections inherent in the accustomed syringe-needle techniques but also decreased the time the services of nurses were needed, thus freeing them for more important duties. It is concluded that the Ampin is the greatest advance in hypodermic techniques since the introduction of the syringe and needle.

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SOURCES OF ERROR IN ENZYMATIC HISTOCHEMISTRY

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ALTHOUGH histochemical techniques for hydrolytic enzymes have become routine tools in biologic research and usually give consistent results in the hands of most workers, occasionally conflicting results are reported with their use. An attempt will be made to explain some of the inconsistencies and to show ways to avoid them. In addition, a fallacy in the quantitative interpretation of the pictures obtained will be pointed out. Most of the examples will be taken from the technique for alkaline phosphatase.

Erroneous results will be discussed under three headings: (a) false negative reactions, (b) false positive reactions, (c) false localization.

(a) False Negatives.—A false negative reaction is defined as a failure to get a positive reaction in spite of the known presence of enzyme in the tissue examined.

Inactivation of the enzyme by grossly inadequate treatment of the tissue such as fixation in unsuitable media will not be considered since it is unlikely to occur in expert hands. Inactivation on mere standing of paraffin blocks has been reported by Lison[1] but could not be confirmed in this laboratory. Paraffin sections stored at room temperature for over five years were found to be just as active as fresh ones. Blocks stored for over ten years yielded sections which stained entirely satisfactorily. Lison also mentions that only desmoenzymes will be demonstrated because lyoenzymes will diffuse out of the section during incubation. While the possibility of some loss by diffusion cannot be ruled out, it is not likely to be a factor of great importance since slides soaked in distilled water or in an alkaline buffer for over twenty-four hours give results indistinguishable from those obtained with fresh sections although sometimes incubation time must be extended 50 to 100 per cent. Even if loss by diffusion would be a serious problem, it could be prevented by coating the slide with dilute collodion; the large molecules of the enzyme will not diffuse through it. However, this protection with collodion may lead to complications because some of the substrates (nucleic acids, certain Tween-like compounds) either do not penetrate it at all or do so only with difficulty.

One of the reasons for a false negative reaction is too feeble (subthreshold) activity of the tissue. Like all chemical reactions, histochemical techniques also have their limits of sensitivity; in the case of alkaline phosphatase this is in the neighborhood of 25 μM-units (about 1 Bodansky unit) per gram of reacting structure, the time of incubation being one hour.[2]

Conditions unfavorable for precipitation often prevent a visible reaction even in an otherwise highly active tissue. It was pointed out in a previous paper[3] that the rate of production of phosphate ions must exceed that of diffusion; otherwise the solubility product of the desired precipitate cannot be exceeded locally. Anything increasing the rate of diffusion (temperature, currents in the incubating medium) or solubility (too low pH, too high an ionic strength) or decreasing the rate of phosphate production (nonoptimal pH or substrate, the presence of inhibitors) may lower the sensitivity of the method to a point where no reaction is obtained in spite of the presence of large amounts of enzyme.

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(b) False Positives.—A false positive reaction is defined as one misleadingly similar to the genuine reaction but not due to enzyme contained in the tissue.

The simplest mistakes may result from confusing blackish pigment with a positive reaction. Hemosiderin, for instance, is converted by ammonium sulfide into green-black FeS. Although the shade of this compound is quite different from the gray-black shade of CoS or from the brown-black one of PbS, errors from this source are possible. To avoid them, in case of doubt a control section should be treated with acidified ferrocyanide; iron will show up in the shade of Prussian blue.

Since preformed calcifications in tissues are essentially identical in composition with the product of enzymatic hydrolysis, it is obvious that they will also be converted into CoS, PbS, or Ag in the course of the technique. The best way to avoid this source of error is to remove preformed deposits of lime salts by treating the slide for five to ten minutes with a 0.1M citrate buffer of pH 4.5 to 5.5. This treatment does not harm the enzyme.

Ruyter and Neumann[4] have reported spurious reactions from the precipitation of some Ca compound from substrate-free incubating mixtures. Their findings could not be duplicated in this laboratory. Sections were incubated in buffer solutions ranging from pH 8 to 10.5, in the presence of 0.1 to 1.0 per cent of $CaCl_2$, and in no instance was any reaction obtained.

Occasionally, Co is adsorbed on certain tissue elements[5, 6] (muscle, coarse connective tissue fibers, colloid) and may show up in a pale yellowish-brown shade, not likely to be mistaken for a genuine enzymatic reaction. The errors due to absorption of Pb on nervous elements in the course of the technique for acid phosphatase are well known.[7, 8] They can be avoided by inactivating a control slide (by Lugol's solution or by 0.1N mineral acid) and incubating it together with the test slide. Only the difference between the two slides is due to enzymatic activity.

In the case of certain substrates (adenosintriphosphate, acyl phosphates, phosphamides) spontaneous hydrolysis of the substrate may lead to indiscriminate precipitation of Ca or Pb phosphate all over the slide. To a great extent this can be prevented by supporting the slides in an inclined position, with the section facing downward. However, in such instances great care must be exercised in the interpretation of results, the more so as sometimes a selective precipitation at sites, similar to those to be described, is encountered.[9]

Bacterial hydrolysis of the substrate is a rare complication if proper precautions are taken (use of chloroform or camphor in the incubating medium) but it will occur once in a while, especially when incubating time is greatly prolonged. The incubating medium may become more or less turbid either by the growth of microorganisms or by supersaturation with Ca phosphate, formed by bacterial phosphatase. On chemical analysis, such fluids contain large amounts of free phosphate. Peculiar false positive reactions are observed in such instances: nuclei are usually quite intensely stained; muscle and connective tissue fibers and calcified tissues such as bone spicules (after previous decalcification with citrate) may be stained in shades of gray to black.

(c) False Localization.—This will be defined as a positive reaction due to enzyme contained in the tissue itself but appearing at sites other than the exact primary microscopic localization. Theoretically, such reactions may be due to two causes: (1) diffusion of enzyme from the original site and its adsorption elsewhere; (2) diffusion of products of enzymatic hydrolysis and their precipitation at sites away from those of their formation. The first possibility, although not considered likely for reasons mentioned before, was tested experimentally (Experiment 1). The deposition of products of hydrolysis requires much more consideration. The selective affinity of certain structures to small molecules, resulting in adsorption from even extremely dilute solutions, is exemplified in spurious reactions for iron in nuclei and in calcified material.[10, 11] In the case of saturated and, even more, of supersaturated solutions, the solute may settle out at sites favorable for deposition, while other sites may be completely spared. An example of this is seen in

in vitro calcification,[12, 13] when purely inorganic media are used (Robison's "second factor"[14]). Typical instances of false positive reactions due to microbial hydrolysis and gradual supersaturation of the incubating medium with Ca phosphate were mentioned previously. Identical results can be obtained under sterile conditions, with stable substrates, if a small amount of phosphatase solution (20 to 100 μM-units to a Coplin jar) is added to the incubating mixture, and inactivated slides are kept in it until the appearance

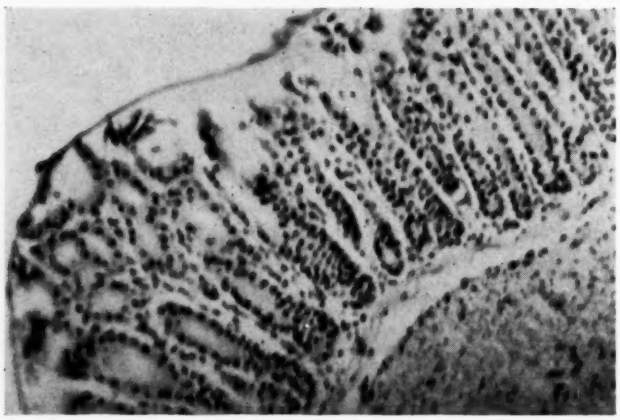

Fig. 1.—Inactivated section of rat stomach, incubated in the presence of 50 μM-units of phosphatase at pH 7.8 for five hours. Intense staining of nuclei (compare with Fig. 3 in Dempsey and Deane's paper[23]).

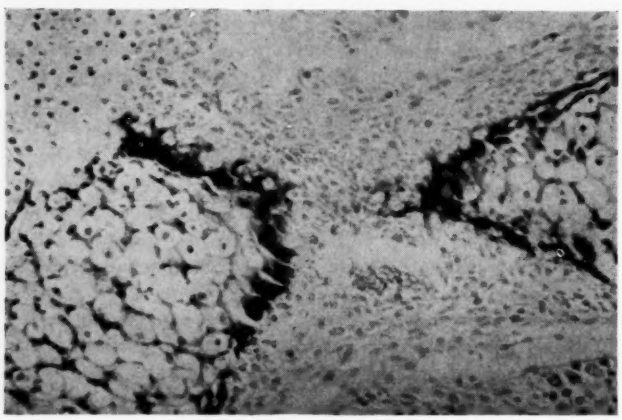

Fig. 2.—Inactivated section of a rat embryo, treated as the previous section. Intense staining of calcified matrix.

of a barely visible haze (one to eight hours, depending on the amount of enzyme added and on the pH of the mixture). The most striking results are secured in the pH range between 7.2 and 8.4 (Figs. 1 and 2); both above and below this range the intensity of the false reaction rapidly decreases. It will be shown in the experimental part of this paper that enzyme contained in the incubated tissue proper may produce similar artifacts.

An interesting type of artifact is met with in Nachlas and Seligman's new technique[15, 16] for esterase. This method is based on the use of naphthyl acetate as a substrate, in the presence of a diazonium salt. The naphthol liberated couples with the diazonium compound to form intensely colored and insoluble azo dyes. It should be remarked that the topography of enzymatic activity as seen with the new method is not

identical with that obtained by the Tween technique.[17, 18] Among others, certain cells of the cardiac portion of the mouse stomach and the cellular debris in the tubules of the mouse testis are intensely positive with Tweens as substrates and negative with the naphthol technique; whereas the superficial cells of the gastric mucosa and the interstitial cells of the testis stain much more intensely with the new technique than with the old one.[19] These findings seem to indicate the existence of several different esterases. However, the authors of the new method describe a few localizations of activity which could not be verified in this laboratory when more insoluble azo dyes were produced and the amount of acetone was reduced to a minimum. These localizations are as follows: the parietal cells of the stomach of man, the dog, and the rabbit; leucocytes of the dog; elastic fibers in vessel walls. These sites were found invariably and completely devoid of activity with both the Tween and naphthol techniques. However, they could be stained, as will be shown in the experimental part, when diffusion artifacts were produced on purpose.

EXPERIMENTAL PROCEDURE AND RESULTS

Paraffin sections of various human and animal tissues, fixed in chilled acetone, were used. Every block contained a number (five to twenty) of different tissues of one species.

Experiment 1. Adsorption of Enzyme on Tissues.—Sections were inactivated with Lugol's solution or, even preferably, with 0.25 per cent HNO_3. Sections treated in the latter way did not show the slightest traces of any reaction, due either to enzymatic activity or to preformed lime salts, even after

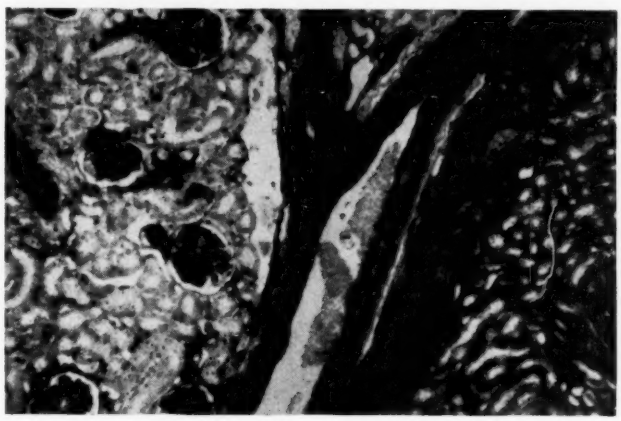

Fig. 3.—Inactivated section of dog kidney, exposed to a solution of purified alkaline phosphatase (4,000 μM-units per milliliter). Glomeruli, vessel walls, connective tissue of renal medulla intensely stained; renal tubules unstained.

prolonged incubation in the substrate mixture. They were subsequently placed in solutions of purified alkaline phosphatase, containing 750 to 15,000 μM-units of enzyme per milliliter, for ten to twenty minutes and then thoroughly washed under the tap. They were then dehydrated with alcohol, protected with collodion (to prevent further diffusion of any adsorbed enzyme), and incubated just as regular slides are. Intense and typical reactions were obtained with all slides exposed to solutions with an activity of 2,000 μM-units or more; however, the distribution of the reaction was utterly different from that seen in regular control sections. The normal sites of activity were completely negative; instead,

a number of structures, negative under normal conditions, such as smooth muscle of viscera and vessel walls, and the connective tissue of the renal medulla, were intensely stained (Fig. 3). A small number of nuclei, scattered in an apparently haphazard way, also showed a reaction while the overwhelming majority of the nuclei remained entirely negative.

Experiments 2, 3, and 4. Artifacts Resulting From Adsorption of Products of Hydrolysis.—

Experiment 2: An active slide and an inactive one were turned face to face to each other. The two ends of the slides were separated by a strip of celluloid film, and the slides were fastened to each other by rubber bands. In this way a space of about 0.2 mm. in width was maintained between the slides.

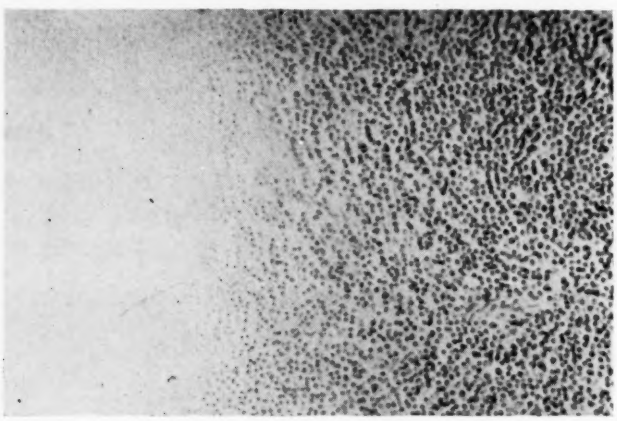

Fig. 4.—Inactivated slide of rat spleen, incubated face to face with active slide. In area opposing active tissue, intense nuclear staining; no nuclear staining outside this area.

The pairs were incubated at various pH levels for various lengths of time. Between pH 7 and 8.5, whenever an intense reaction was obtained in the active slide, a distinct dark gray staining with somewhat fuzzy outlines was seen in the opposing areas of the inactivated section. Microscopically, around areas of high activity in the active slide, there was an intense blackening of the nuclei; in the gray areas of the inactivated slide a very similar nuclear staining was observed (Fig. 4); whenever bone matrix was present, it was stained even darker. Outside the pH range mentioned, this "imprint" type of reaction was weak or absent.

Experiment 3: (Idea of Danielli,[9] modified.) A number of sections from a paraffin ribbon were inactivated by floating them for five minutes on 0.25 per cent HNO_3 and then were refloated on a buffer of pH 9.2 and finally on distilled water. Subsequently they were mounted on top of sections of known high activity. Slides carrying the combined sections were carefully deparaffinized, protected with collodion, and incubated as in the previous experiment. Between pH 7 and 8.5, the inactivated tissue showed nuclear staining within a strip, 10 to 50 μ wide, around centers of high activity in the active section (Fig 5). At pH 9.2, no sign of diffusion could be noted although Jacoby and Martin[20] did find evidence of diffusion artifacts even between pH 9.2 and 9.5.

Experiment 4: Five to seven slides, carrying fairly large and active sections, were incubated in one Coplin jar with one or two inactivated ones for twenty-four to ninety-six hours. Again, in the pH range mentioned before, a distinct nuclear staining was observed in the inactivated slides whenever incubation was extended beyond thirty-six hours. No staining whatsoever was obtained when inactivated sections were incubated by themselves.

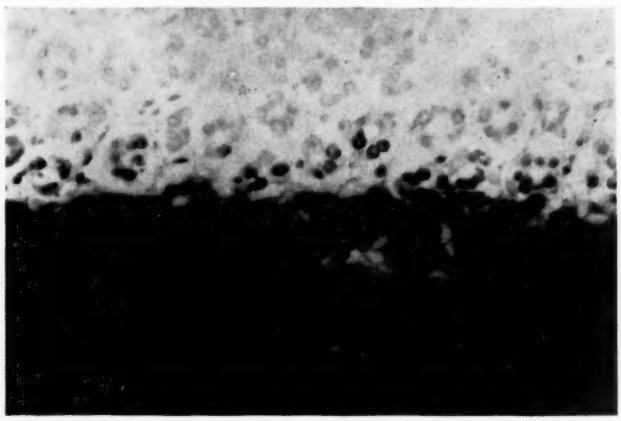

Fig. 5.—Inactivated section of rat stomach, superimposed on an active section of an osteogenic sarcoma. Scattered nuclei stained in proximity of active tissue.

Experiment 5. Production of Artifacts in the Naphthol Method for Esterase.—In preliminary test tube experiments it could be shown that azo dyes produced by coupling alpha naphthol with various diazonium compounds around pH 7 will precipitate instantly, while part of the azo dye produced with beta naphthol will remain in solution for many minutes, especially in the presence of more than 1 per cent of acetone. In histochemical experiments, whenever beta naphthyl acetate was used as a substrate, especially when acetone in excess of 1 ml. was added to a Coplin jar, clouds of the shade of the azo dye were seen floating around the section after about fifteen minutes of incubation, and at the same time the sites mentioned became stained. An even more selective staining of the same structures was obtained when slides were incubated with very dilute solutions (2 to 10 mg. in 50 ml.) of either alpha or beta naphthol for a few hours, washed thoroughly under the tap, and treated with the diazonium compound. On the other hand, when alpha naphthyl acetate was used as a substrate, and the amount of acetone was cut down to 0.5 ml. per Coplin jar, much sharper pictures were obtained with no staining at all of the anomalous sites.

DISCUSSION

Since the type of reaction described in Experiment 1 is obtained only after exposing slides to highly concentrated solutions of enzyme and is never seen in normal sections, false localizations due to adsorption of enzyme can be disregarded as a practical problem. On the other hand, the positive reactions obtained in Experiments 2, 3, and 4 clearly indicate that the deposition of

products of hydrolysis, resulting either in false positive reactions or in false localizations, depending on whether the enzyme comes from the outside or from the tissue proper, is a truly important source of error. In Experiments 2 and 3, short-range effects (up to a distance of a few hundred micra from centers of activity) were shown; in Experiment 4, the existence of long-distance effects was proved.

The results of Experiment 4 are in good agreement with those expected on the basis of theoretical considerations.

The solubility product of $CaHPO_4$ at pH 7.4 and at an ionic strength of 0.16 is about 3.3×10^{-6}.[21] Since the concentration of Ca ions in the average incubating medium is 0.01 to 0.02M, the solubility product is reached at a free phosphate concentration of 0.00015 to 0.0003M, i.e., at a free phosphorus content of 0.25 to 0.5 mg. per Coplin jar. A tissue section measuring 12 by 12 by 0.007 mm. and weighing about 1 mg. may contain as much as 0.02 Bodansky unit of enzyme in the case of highly active tissue (rat kidney: about 20 units per gram). This amount of enzyme will produce about 0.4 mg. of P in twenty-four hours, enough to saturate the incubating medium. Naturally, activity was calculated for pH 9.2; at pH 7.6 it would be much lower (10 to 35 per cent, depending on the source of the enzyme[22]); but often the amount of tissue per Coplin jar is much more than 1 milligram. Also, at pH 9.2 Ca phosphate is very insoluble and will precipitate instantly, as formed, producing pictures corresponding to true localization. Around pH 7.4 it is much more soluble and will not precipitate locally (at least, not completely) unless produced at a very fast rate; instead, it will diffuse into the surrounding medium. If and when the solubility product is reached, it will precipitate at certain sites where the constitution (physical or chemical or both) of the matrix is favorable. This is most likely to occur with sugar phosphates as substrates, the commercially available brands of which usually contain fairly large amounts of free phosphate, while glycerophosphate, as a rule, is quite pure.

The experiments presented clearly show that false localizations can be obtained whenever sections are incubated for days at a pH below 8.5. The structures stained most deeply are nuclei and bone matrix. Reports on nuclear activity after long incubation close to the neutral point[6, 23] should be re-evaluated in the light of these findings.

Experiment 5 proves that the staining of the anomalous sites in the naphthol technique for esterase is not due to local enzymatic activity but to the selective adsorption of either free naphthol or of the azo dye formed by enzymatic action elsewhere.

A few remarks will be made on the quantitative interpretation of results. Whenever more or less diffuse blackening of the tissue in the course of the enzymatic reaction is found, this finding is often reported in terms of an "intense reaction" or "large amounts of enzyme." It should be made clear that a widespread reaction does not necessarily mean high enzymatic activity per gram of tissue. A quantitative estimation of activity is possible only by means of laborious comparative experiments, standards of known activity being used for comparison.[2]

SUMMARY AND CONCLUSIONS

The main errors in enzymatic histochemistry are false negative reactions, false positive reactions, false localization, and false quantitative evaluation of results.

The use of proper precautions will reduce errors due to false negative and positive reactions to a minimum.

False localization is almost invariably due to supersaturation of the incubating medium with products of hydrolysis. Too long incubation of slides at low pH values is the most frequent cause of artifacts.

Quantitative evaluation of results can be done only on the basis of comparison with known standards.

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A UNIVERSAL SYRINGE BURET AND ITS USE IN MICROCOLORIMETRIC ANALYSIS

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INTRODUCTION

A MICROBURET suitable for titrating one drop of liquid was designed by Rehberg in 1925.[1] Since that time many modifications have appeared. In general, the volume of liquid delivered was measured in a calibrated capillary tube and mercury was usually used to expel this liquid. In a modification by Scholander,[2] a machinist's micrometer was used to expel the fluid and the volumes delivered were read directly on the micrometer scale. In a later modification by Gilmont,[3] a dial indicator gauge was substituted for the micrometer and the reading of the scale was further facilitated. However, since most of these burets use mercury to expel the fluid from the buret, they cannot be used with solutions that react with mercury. Furthermore, the cleaning of a buret that contains mercury is a time-consuming operation.

In 1925, Treven[4] employed a micrometer syringe for measuring small quantities of fluid. A glass tip was fused to a hypodermic syringe and a micrometer was used to advance the plunger. By completely filling the syringe with the reagent, an air-free system was obtained and precise volume measurements could be made without the use of mercury. Similar syringe burets were also used by Krogh and Keys[5] and Clark and co-workers.[6]

The syringe buret herein described consists of a Universal syringe holder which will center syringes ranging in capacity from ¼ to 10 cubic centimeters. The syringe is attached to a buret tip by means of a standard taper joint and the latter is therefore easily replaceable. A dial displacement gauge is used to advance the plunger of a syringe of standard diameter, and the scale divisions of the displacement gauge correspond to an even number of microliters. Since the syringes may be easily and quickly interchanged, many different syringes may be used in the same apparatus.

APPARATUS

The apparatus* consists of a cast aluminum stand, Fig. 1, *A*, which includes three vertical bars, *B*, *C*, and *D*. Eight V-shaped grooves, *E*, are cast into the stand and they hold the syringe burets which are not in use. A machinist's dial displacement gauge, *F*, is mounted on bar *C*. This gauge has an adjustable scale which can be set at zero for any position of the needle, *G*, and locked in place by means of screw *H*. Each small scale division as indicated by the large needle, *G*, corresponds to a linear displacement of .001 inch. Each scale division indicated by the small needle, *I*, corresponds to a displacement of 0.1 inch. Thus the total displacement of the gauge is 1 inch and displacements as small as

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*This apparatus is available through the Micrometric Instrument Co., 7629 Kinsman Ave., Cleveland 6, Ohio.

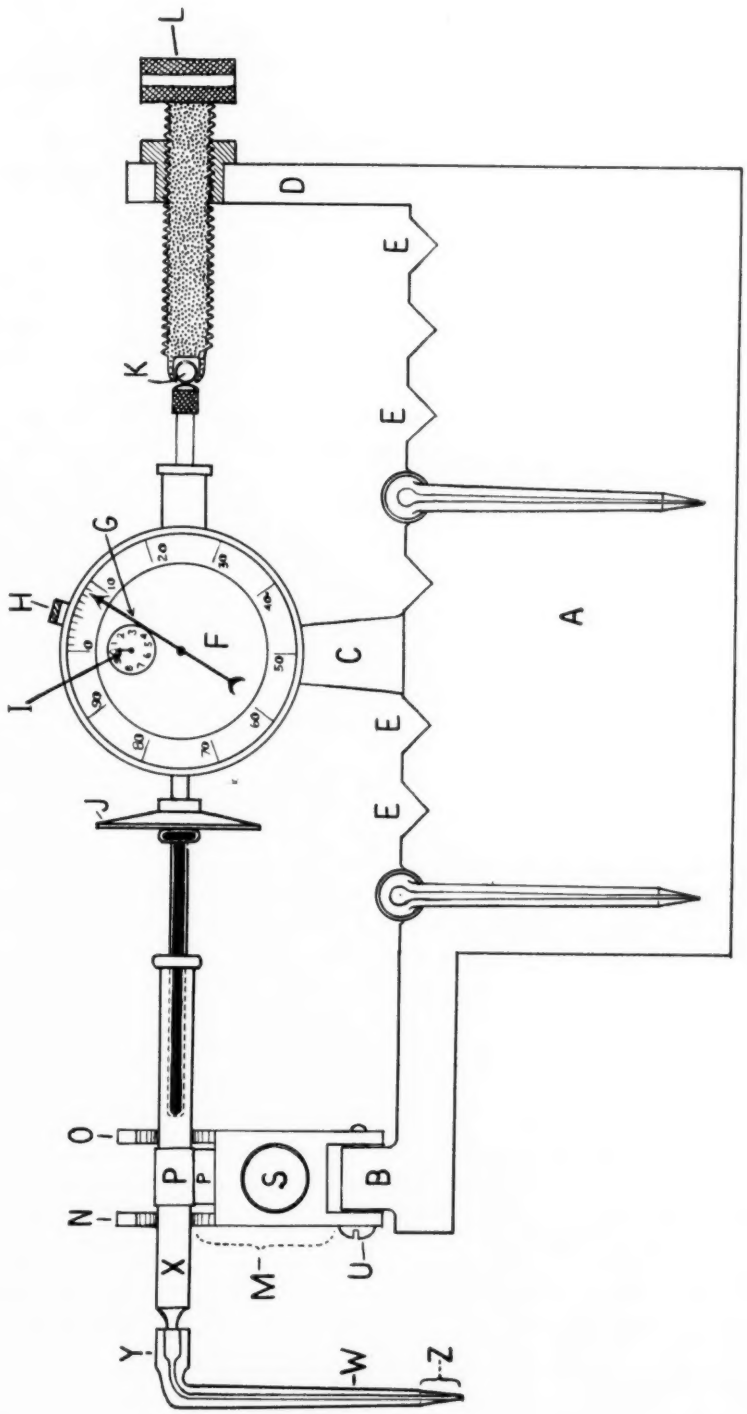

Fig. 1.—Syringe buret. See text for explanation.

.0001 inch may be interpreted from the scale. A face plate, *J*, is pressed on one end of the displacement gauge and it is used to advance the plunger of the syringe. A ball bearing, *K*, is inserted into the end of screw *L*, which is in turn mounted on the vertical bar *D*. This screw advances the dial indicator gauge through the precision ball bearing, *K*. The syringe is mounted on the Universal holder, *M*, which is in turn mounted on the vertical rod *B*. The barrel of the syringe is held firmly in place between the V grooves of the vertical bars, *N* and *O* (see also Fig. 2), by a spring brass band, *P*. Both ends of this brass band, *P*, are

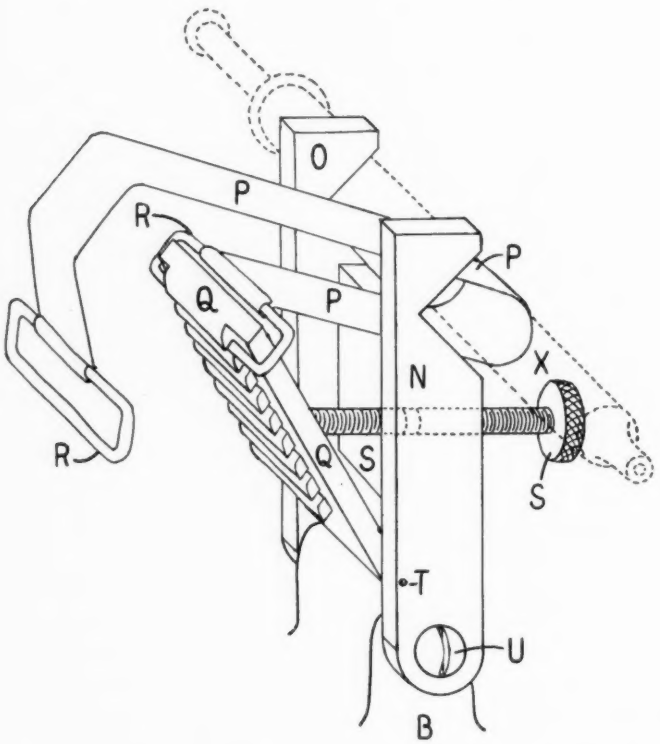

Fig. 2.—Universal syringe holder. See text for explanation.

attached to a plate, *Q*, by wire loops, *R*. A number of grooves milled on the back of plate *Q*, permit most of the slack of band *P* to be taken up. The syringe is firmly attached to the holder by tightening screw *S*. Since plate *Q* is pivoted at point *T*, tightening of the screw, *S*, removes all of the slack in band *P* and the syringe barrel is held firmly in place. Because of the V shaped groove in bars *N* and *O*, syringes of all diameters will be centered in a vertical plane, with respect to the dial displacement gauge. However, the center of the syringe is usually not in alignment with the horizontal axis of the dial indicator gauge. To accomplish horizontal alignment, the Universal holder is rotated on the axis *U*, which is the screw that attaches the holder, *M*, to the vertical block *B*. Thus syringes of widely varying diameters may be quickly aligned and centered. Failure to align the syringe with the axis of the displacement gauge will cause binding of the syringe plunger upon advancement of the displacement gauge.

The buret tip,* *W*, Fig. 1, is attached to the syringe, *X*, by a standard taper joint, *Y*, and the tip of the buret, *Z*, is ground to a fine, tapering point.

*Available from Becton, Dickinson and Company, Rutherford, N. J.

Standard Diameter Hypodermic Syringes.—Inasmuch as each small scale division corresponds to .001 inch, it is possible to make these scale divisions correspond to an even number of microliters by selecting syringes of appropriate diameter. Table I lists the requisite plunger diameters for the various syringes used.

TABLE I

TYPE OF SYRINGE	VOLUME OF SYRINGE (C.C.)	PLUNGER DIAMETER (IN.)	VOLUME DISPLACEMENTS		
			C.C. PER IN.	μL PER 100 DIVISIONS (.100 IN.)	μL PER DIVISION (.001 IN.)
Tuberculin	¼	.125	.200	20	0.200
Tuberculin	1	.197	.500	50	0.50
Tuberculin	2	.279	1.00	100	1.00
Regular	5	.483	3.00	300	3.00
Regular	10	.624	5.00	500	5.00

Syringes of appropriate diameters are manufactured by Becton, Dickinson and Company. Each syringe is engraved with its volume displacement per inch. In general it is desirable to choose the syringe diameter so that 50 to 100 scale divisions correspond to the requisite volume to be delivered. It has been found that by using a ¼ c.c. tuberculin syringe, which has a volume displacement of 0.2 c.c. per inch, volumes as small as 2 μl* may be delivered with an accuracy of 1 to 2 per cent, even though only ten small scale divisions are used.

Calibration of the Syringe Buret.—The calibration of the buret may be checked by using the India ink dilution method.[7] In this procedure 2 to 500 μl of a standard solution of India ink are measured into a 5 or 10 c.c. volumetric flask. The concentration of the standard ink solution is chosen so that after final dilution this solution can be read on a Klett colorimeter† and compared with standard dilutions of India ink prepared by ordinary volumetric procedures. It has been found that the volume displacement for each 0.1 inch is constant for the entire length of the syringe.

RESULTS

Use of Syringe Burets in Microcolorimetric Analyses.—Lowry and Bessey[8] devised a microcell for the Beckman spectrophotometer which requires between 50 and 200 μl of fluid. Since these cells have an optical path of 10 mm., they give the same extinction value as is obtained with the macro Beckman cells. Therefore, microcolorimetry may be carried out by merely reducing the volumes of all the reagents used by a factor of 50 or more. In carrying out microcolorimetric analysis, various types of micropipettes have been used.[9-11] However, the syringe buret herein described is ideally suited for this purpose. Each reagent is delivered by an appropriate syringe.‡ If possible, the diameters of the various syringes are chosen so that 100 scale divisions (0.1 inch) corresponds to the volume which is to be delivered, i.e. 20, 50, 100, 300, or 500 microliters. The reagents are delivered into micro test tubes 10 by 80 millimeters. One filling of the syringe buret is sufficient for at least nine determinations. The

*When using this buret to deliver these small volumes, it is desirable to paraffinize the tip of the buret to prevent adherence of liquid to the buret tip.

†When Higgins' soluble India ink is diluted 5,000 times with dilute ammonia water it gives a reading of about 300 in a Klett colorimeter using a 420 filter.

‡The spring clip (not illustrated) ordinarily supplied with the hypodermic syringe restricts the movement of the plunger in the barrel. It is usually removed before attaching the syringe to the buret stand. Occasionally, however, the liquid in the syringe will very gradually drip out of the buret tip and the plunger advances without movement of the screw, *L*. This spontaneous drainage is due to the hydrostatic pressure developed by the liquid column in the buret tip and it may be prevented by using the spring clip ordinarily supplied with the syringe.

interchange of the syringes for the delivery of subsequent reagents is rapidly accomplished. Following the completion of the colorimetric reactions, the contents of the tube are transferred to the Beckman microcells by means of a finely drawn eye dropper and the extinctions determined. The accuracy of the determination as carried out by the microprocedure using the syringe buret is about the same as for the macromethod. However, the microdeterminations may be carried out with much greater rapidity and they require much less table space and reagent.

The syringe buret may be used for the conventional microtitration but we have found it has a much wider application in microcolorimetric analyses. The cost of the preselected syringes is reasonable and therefore one buret stand can be made equivalent to a battery of microburets.

SUMMARY

A syringe buret is described which has the following advantages: (a) Readings are made on a dial indicator gauge. (b) The use of mercury is avoided and the cleaning of the buret is therefore greatly simplified. (c) The syringes may be rapidly replaced, and a wide variety of sizes may be used ranging from a 1/4 c.c. tuberculin to a 10 c.c. syringe. (d) Syringes of preselected diameters are used so that the scale divisions correspond to an even number of microliters.

The syringe is admirably suited for microcolorimetric analyses.

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DILUTION AS A SOURCE OF ERROR IN CHYLOMICRON COUNTS

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THE chylomicron count has become an increasingly popular method for studying fat absorption following the administration of a fatty meal.[1-7] In carrying out this method, the lipemic sera present two to six hours after the meal are sometimes diluted to facilitate counting of the closely packed lipid particles.

Such dilution has yielded grossly inaccurate results in our laboratory. As shown in Table I, serial dilution of a given sample of serum produces a progressive increase in the calculated chylomicron count for the sample. This effect may be the result of an actual change in the number and size of the visible particles in Brownian movement or it may be due merely to dispersion. As the distance between particles increases, more of the smaller and duller particles may become visible, whereas only the larger and brighter ones are seen when the particles are closely packed.

The chylomicron count, difficult at best because of errors introduced by minor changes in the adjustment of the microscope and source of light, appears to be completely invalid when selected samples are diluted. As shown in Table I, however, a given dilution tends to increase the counts by a relatively constant factor, irrespective of the number of chylomicrons in the undiluted sample. A 1:20 dilution, for example, generally increases the count of the undiluted sample three- to fourfold. For this reason, chylomicron counts carried out on serial blood samples obtained after a fatty meal may yield results of relative significance provided all samples are diluted to the same degree.

TABLE I

	SAMPLE									
DILUTION	1	2	3	4	5	6	7	8	9	10
Undiluted	596	560	389	186	227	181	768	525	45	466
1:2	799	735	497	205	309	229	1,076	748	74	555
1:3	1,076	882	582	206	378	357	1,152	798	138	671
1:5	1,117	1,135	798	308	557	412	1,313	990	251	833
1:10	1,840	1,510	1,313	540	607	580	1,907	1,537	250	1,147
1:20	2,193	1,893	1,360	953	827	740	2,627	1,760	314	1,293
Turbidity	.056%	.043%	.031%	.028%	.028%	.016%	.057%	.042%	.023%	.057%

Figures represent chylomicron counts per oil immersion field (average of 3 fields) multiplied by dilution factor. Turbidity measurements were carried out on the original sample by a modification of the method of Geyer, Mann, and Stare.[8]

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A METHOD OF CHEMICAL ANALYSIS OF GUINEA PIG LUNG FOR THE FACTORS INVOLVED IN PULMONARY EDEMA

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INVESTIGATIONS on the pathogenesis of pulmonary edema have received considerable attention in recent years because of the occurrence of pulmonary edema in heart disease, poisoning by war gases, and in bulbar poliomyelitis. In this last condition, pulmonary edema following bulbar paralysis is usually the immediate cause of death. In order to evaluate the various forms of treatment for this disease, and in an attempt to produce a rational therapy based on pathogenesis, a study of pulmonary edema and its various forms is being made. A review of the published papers on pulmonary edema will reveal that the methods for estimating the degree of edema have been qualitative rather than quantitative. It has been customary to extirpate a lung at autopsy, note the color and the presence of hemorrhagic areas, look for fluid in the air passages, and determine the consistency by palpation. These methods, while quite adequate for distinguishing between a normal lung and one that is grossly edematous, do not serve to distinguish mild edema, nor do they offer a quantitative numerical evaluation of the degree of injury. The sole quantitative measurement has consisted of a determination of lung weight, and once the relation between lung weight and some other physical magnitude, e.g. body weight, is known then it is possible to estimate what the "normal" lung weight should have been had that particular animal been normal, see Winter,[1] Stone and Loew,[2] and Wood and Moe.[3] In order to improve methods for determining edema in lungs and in order to obtain quantitative information on factors pertinent to edema other than lung weight, a method for analysis of edematous and normal lungs has been devised.

The edematous lung is enlarged and congested because of the influx of blood and edema fluid. The lung parenchyma in pulmonary edema is not increased but the lung is expanded by the invasion of fluid. The protein in the edema fluid is soluble, consisting of plasma protein. During the edema, therefore, the parenchymal protein is not increased. Hence an analysis made for the insoluble pulmonary nitrogen is a measure of the structural and functional tissue of the lung. The procedure which is used consists of rapidly extirpating the lung after sacrificing the animal by guillotine. After weighing and measuring the volume the lung is ground to a paste in sand. The ground residue is extracted several times with 1 per cent NaCl solution which removes the soluble proteins including hemoglobin. An aliquot of the saline extract is used for spectrophotometric hemoglobin determination and another aliquot is used for determination of the total soluble protein nitrogen. From this measurement the soluble

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nonhemoglobin protein nitrogen can be determined by subtracting from the total soluble proteins which the supernatant fluid contains a nonprotein nitrogen fraction. Hence, in addition to weight this analysis of the normal or edematous lung gives (1) the lung volume (or density), (2) the insoluble protein nitrogen, (3) the pulmonary hemoglobin, (4) the soluble nonhemoglobin protein nitrogen, and (5) the pulmonary nonprotein nitrogen. A determination of the pulmonary hemoglobin gives a quantitative measure of what has been previously designated as pulmonary "congestion." The soluble nonhemoglobin protein nitrogen is a measure of the plasma protein which has invaded the lung as a result of the edema. This method has been investigated, a number of refinements have been made, and certain difficulties have been eliminated. The method finally evolved after a number of experimental trials is free from difficult or elaborate procedures, and accurate data can be obtained by a well-trained chemical or medical technician.

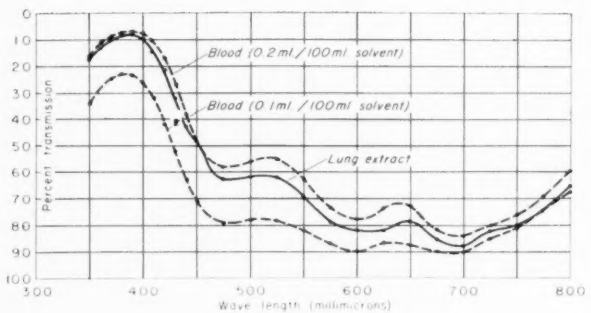

Fig. 1.

METHODS

Measurement of Pulmonary Hemoglobin.—One of the chief difficulties encountered in this method was the determination of pulmonary hemoglobin. After grinding the lung in sand and extracting soluble protein in 1 per cent NaCl, the extract is always turbid and hemoglobin cannot be determined directly on the extract because of this turbidity. A method has been devised consisting of a procedure resembling that of Flink and Watson[4] and Greenberg and Erickson[5] but modified in certain details. The present procedure consists of taking an aliquot of the saline extract and drying it in a vacuum desiccator. To the dry residue a solvent containing alcohol, ether, acetic and hydrochloric acid is added and this mixture extracts the acid hematin in a clear solution suitable for colorimetric determination. (Before extracting with the acid-alcohol-ether mixture, an acetone extraction of the dried residue can be made and this was done in the earlier modifications of the method. It has been found, however, that this preliminary acetone extraction is unnecessary.) After adding the acid-alcohol-ether solvent, the dried residue is loosened from the evaporating beaker with a glass rod, pulverized with a homogenizer, and transferred to a centrifuge tube. After centrifugation the clear but colored supernatant fluid is analyzed for acid hematin with the spectrophotometer. In order to determine whether or not the color of the pulmonary extract was due to hemoglobin, a comparison was made of the absorption spectra of the pulmonary extract and two samples of guinea pig blood extracted in the same manner as the lung. The absorption spectra of the pulmonary extract and the guinea pig blood are given in Fig. 1. The close resemblance of the spectra establishes the identity of the chromogenic substance of the pulmonary extract as hemoglobin.

From an inspection of the absorption spectra of Fig. 1 it is obvious that the maximum absorption peak occurs at a wave length of 390 mμ rather than the wave length of 635 mμ used by Greenberg and Erickson. To determine the amount of pulmonary hemoglobin spectrophotometrically the Coleman model 11 grating spectraphotometer was used with the wave length transmission band set at 390 millimicrons. A sample of guinea pig blood was drawn and its oxygen capacity determined by the Van Slyke manometric procedure. Knowing the oxygen capacity, the hemoglobin content was determined. Samples of 0.1 ml. and 0.2 ml. of the blood were subjected to the desiccation and extraction procedure and the per cent light transmission was determined per centimeter of optical path for a known solvent

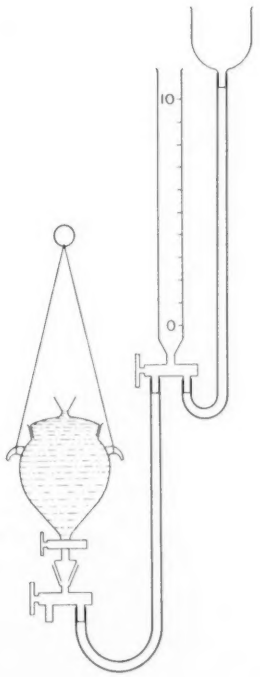

Fig. 2.

volume. From this reading the logarithmic transmission ratio was determined and the Beer's law constant computed. Since the lungs analyzed varied widely in their hemoglobin content, it was customary to dilute the acid hematin extract until the per cent transmission was in the range of 20 to 80 per cent. The color of a hematin solution having this per cent transmission was soon learned by the analyst and thereafter the acid hematin extract was diluted until visual inspection revealed a color of the approximate desired intensity. The dilution being known, the solution was then placed in the Coleman spectrophotometer and the exact per cent transmission (or logarithmic transmission ratio) was determined. Knowing the Beer's law constant and the length of the optical path, the hematin concentration was determined and computed as hemoglobin.

Lung Density.—A densitometer has been devised for measurement of the lung weight and lung volume from which the lung density can be determined. The densitometer is shown in Fig. 2. The densitometer consists of a weighing bottle suspended by a wire and having a wide mouth, a ground-glass stopper, and a ground-glass tapered lower tip which fits into a ground receptacle located on the upper single arm of a three-way stopcock. The stopper tapers upward to a short capillary, above which is a small funnel. A line is etched around the narrowest position of the capillary. One of the lower arms of the three-way

stopcock is attached by a Koroseal tube to a 10 ml. burette which in turn can be filled with 1 per cent NaCl by allowing fluid to flow into the upper arm of the three-way stopcock. The cock is then reversed 180 degrees and this fluid is drawn out by suction. The dry and empty weighing bottle is then attached to the upper arm of the three-way stopcock and the weighing bottle is filled to the mark on the capillary of the cover. The volume of fluid from the burette required to fill the weighing bottle gives its volume. The same procedure is repeated with a guinea pig lung in the bottle and the fluid volume again is measured on the burette. The difference between these two volumes gives the lung volume.

Reagents.—Ether Alcohol Acid Reagent: 5.7 ml. of glacial acetic acid and 8.6 ml. of 37 per cent HCl are added to 800 ml. of ethyl ether (lipid extraction ether) and 200 ml. of ethyl alcohol.

Tungstic Acid: This is freshly prepared when used: 20 ml. of 10 per cent sodium tungstate are placed in a beaker and 15 ml. of $\frac{2}{3}$N H_2SO_4 added. From a burette $\frac{2}{3}$N H_2SO_4 is added until the tungstic acid is just acid to Congo red paper.

Bromcresol Green-Methyl Red Indicator: 5 vol. of bromcresol green reagent (0.1 Gm. in 100 ml., 95 per cent alcohol) are added to 1 vol. of methyl red indicator (0.1 Gm. in 100 ml. of 95 per cent alcohol).

Boric Acid: 60 Gm. of boric acid are dissolved in 3 L. of solution.

Kjeldahl Digestion Mixture: 25 to 30 ml. of fuming sulfuric acid are added to 100 ml. of concentrated C.P. sulfuric acid. Approximately 1 Gm. of anhydrous copper sulfate is added.

Hydrochloric Acid: 0.03N (standardized).

Procedure.—Removal of Lung and Measurement of Density: The guinea pig is killed by guillotine; the chest is opened immediately and the lung removed. Adhering tissue is carefully removed and large vessels are cut off close to the lung surface. The lung is placed on blotting paper for one minute and then transferred to the previously weighed empty weighing bottle shown in Fig. 2. The lung and bottle are weighed and the lung weight is determined by weight difference. The bottle containing the lung is then filled from the burette as described and the lung volume measured. The density is computed from the ratio weight/volume.

Separation of Insoluble Protein: After weighing, the saline from the weighing bottle is decanted and the lungs are transferred to a mortar containing about 8 to 9 Gm. of sand free from organic nitrogen. The lung and sand are ground to a paste and transferred to a 40 ml. heavy-wall centrifuge tube along with the decanted saline. The centrifuge tube is counterbalanced with a tube containing the same amount of sand. The tube is centrifuged and the supernatant fluid decanted into a volumetric cylinder. Saline is added to the residue in the centrifuge tube; the contents are mixed thoroughly and again centrifuged. The washing of the residue with saline is continued until the residue has lost all trace of red color. The volume of the entire saline extract is measured.

Pulmonary Hemoglobin: Two 10 ml. samples of the saline extract are transferred to a beaker; each beaker is placed in a vacuum desiccator and the solutions are evaporated to dryness. To each dried residue 20 ml. of the ether-alcohol-acid reagent are added and the residue is dislodged from walls and floor with a rubber policeman. The contents of the beakers are poured into a ground-glass homogenizer (Wisconsin type) and the suspended particles ground to a finely pulverized state. The turbid solutions are transferred to heavy glass 40 ml. pointed centrifuge tubes, and solvent is added until the volumes are approximately 20 ml.; the tubes are allowed to stand fifteen minutes and then are centrifuged at high speed. The supernatant fluid which is pink because of acid hematin is decanted into a 100 ml. volumetric flask. To the residue in each centrifuge tube, 20 ml. of the solvent are added; the residue is stirred and thoroughly mixed with solvent and the tubes again are centrifuged. This procedure is continued until the insoluble residue is colorless. The collected samples of supernatant fluid should be clear without a trace of turbidity. If turbid, the solutions should be recentrifuged at high speed. The volumetric flask contents are then diluted to 100 ml. with the ether-alcohol-acid solvent. The color of the solution within the volumetric flask is noted and if too intense for the colorimeter an aliquot is removed and diluted with a known amount of solvent until a color suitable for the colorimeter is obtained. The blank

cell of the spectrophotometer contains solvent. The logarithmic transmission ratio at a wave length of 390 mμ is read from the instrument and the amount of pulmonary hemoglobin is calculated from the calibration as previously described.

Insoluble Protein Nitrogen: The solid residue (with sand) remaining after the saline extraction is placed in a macro-Kjeldahl (300 ml.) flask; 10 to 15 ml. of the acid digesting mixture are added with an oxidation catalyst granule and the solution is digested until clear. After digestion the usual Kjeldahl distilling procedure is used to transfer the ammonia into 20 ml. of 2 per cent boric acid. The ammonia distilled is titrated with 0.03N (standard) HCl and the insoluble protein nitrogen calculated.

Soluble Protein (Nonhemoglobin) Nitrogen: Ten milliliters of the saline extract containing hemoglobin, soluble protein, and nonprotein nitrogenous compounds are placed in a special 50 ml. centrifuge tube with a standard taper ground-glass connection at the top, which also functions as a micro-Kjeldahl digestion tube. To the saline extract 5 ml. of freshly prepared tungstic acid are added; the tube is allowed to stand fifteen minutes and then centrifuged. The supernatant fluid containing the nonprotein nitrogen is decanted into a similar micro-Kjeldahl digestion tube. To the protein precipitate and the protein-free Folin-Wu filtrate, acid digestion mixture is added and the tubes are digested until clear. They are then connected by the standard taper joint to a micro-Kjeldahl distilling apparatus. The ammonia released after adding alkali is collected in boric acid and determined by titration. The nitrogen in the protein precipitate is computed and the soluble protein nitrogen of the entire lung determined. From this the hemoglobin nitrogen is subtracted, giving the soluble (nonhemoglobin) protein nitrogen.

Analysis of Normal Guinea Pig Lung.—Table I gives analyses of ten normal guinea pig lungs.

TABLE I

GUINEA PIG	116	118	120	124	126	128	130	132	134	136
Body wt. (Gm.)	260	269	383	181	212	238	216	308	451	460
(Lung wt./body wt.) × 10^4	1.95	2.08	2.30	1.48	1.63	1.61	1.61	2.33	2.59	2.56
	75.0	77.4	60.1	81.8	76.8	67.6	74.5	75.7	57.4	55.7
Lung density	.61	.47	.59	.64	.62	.78	.61	.65	.79	.63
Pulmonary Hb. (mg.)	30.5	38.0	59.5	25.0	22.7	21.0	25.6	24.8	33.3	31.6
Insol. prot. N (mg.)	19.2	15.2	22.2	18.6	15.8	17.0	15.1	21.6	32.6	27.8
Sol. non Hb. Prot. N	17.7	14.5	14.3	12.8	13.8	12.1	13.3	17.8	20.9	22.4
Nonprot. N	4.01	4.65	4.79	3.81	3.36	2.39	2.81	4.14	5.09	4.67

For these ten animals the body weights ranged from 181 to 460 grams, the lung weights from 1.48 to 2.59 grams, lung densities from .47 to .79, pulmonary hemoglobin from 21.0 to 59.5 mg., insoluble protein nitrogen from 15.1 to 32.6 mg., soluble nonhemoglobin protein nitrogen from 12.1 to 22.4 mg., the nonprotein nitrogen from 2.39 to 5.09 milligrams.

SUMMARY

A method has been devised for the quantitative analysis of guinea pigs with a determination of quantities which change significantly when lungs become edematous. The quantities measured include (a) lung weight, (b) lung density, (c) insoluble protein nitrogen of the lung, i.e. nitrogen of insoluble parenchyma, (d) pulmonary hemoglobin, and (e) soluble (nonhemoglobin) protein nitrogen. Values are given for the analysis of ten normal guinea pig lungs.

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THE USE OF ETHYLENE OXIDE FOR THE STERILIZATION OF HOSPITAL EQUIPMENT

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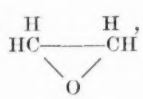
STERILIZATION of hospital and laboratory equipment is most often accomplished by the use of steam under pressure in an autoclave, or by the application of some chemical agent in liquid form. Less commonly employed methods include boiling in water, exposure to various types of radiation, and ultrafiltration. Relatively little attention appears to have been given the possibility of using a bactericidal gas or vapor, although formaldehyde and sulfur dioxide were widely used in the early part of this century to disinfect sickrooms following illness. This practice declined when it was shown that the treatment as carried out was usually ineffective, and probably unnecessary. Much of the interest in disinfecting gases died at this time.

A number of years ago it became necessary to attempt the sterilization of a number of objects which could not be treated successfully by the more common techniques. Articles containing wood, paper, leather, plastics, or wool, as well as foodstuff and delicate metal and glass equipment, presented particularly difficult problems in achieving sterilization without damage to the object being treated.

After a considerable number of gases had been examined, it was found that ethylene oxide vapor was a promising sterilizing agent, for it would diffuse through many types of porous substances, could readily be removed by aeration following treatment, was convenient to handle, and would effectively destroy all forms of microorganisms at ordinary temperatures. Experiments to determine the applicability and the limitations of this compound were performed; in the course of this work, references to the use of ethylene oxide as a sterilizing agent were found in certain trade journals and patent applications.

MATERIALS AND METHODS

The results of the experiments and a complete review of the literature on the properties of ethylene oxide have been published recently,[1-4] so that only a brief summary of these features will be given here.

Ethylene oxide,  , is a three-membered, oxygen-containing cyclic compound,

and by virtue of this structure is extremely reactive. It is widely used as an intermediate in chemical synthesis and is readily available, even in tank car quantities, in the United States. Since the liquid boils at 10.8° C., ethylene oxide is a gas at ordinary temperatures

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and pressures, although it is readily liquefied, and is obtainable as a liquid both in small cylinders and in sealed glass bottles. The liquid is readily stored in small amounts in tightly capped bottles in a dry ice chest. Storage in an electric refrigerator should not be attempted for this liquid (or, for that matter, for any volatile inflammable material), since a small leak might form an explosive mixture which may be ignited by the motor.

Ethylene oxide is vigorously flammable, and the vapors form explosive mixtures with air in all proportions from 3 to 80 per cent by volume. Whenever the pure material is handled, this potential danger must be kept in mind. The explosion hazard is eliminated, however, when ethylene oxide vapor is mixed with more than 7.15 times its volume of carbon dioxide.[5] A commercial product, which contains 10 per cent ethylene oxide and 90 per cent carbon dioxide, is distributed for fumigating use because of its insecticidal activity. This mixture is sold under the name of Carboxide* and can be released into the air in any proportion without forming an explosive mixture. When obtained in 60-pound quantities in cylinders, Carboxide costs approximately 25 cents per pound.

The experiments which are being reported here were performed with bedding obtained from the University of Chicago Clinics, using a simple improvised exposure chamber and a cylinder of Carboxide kindly furnished by the manufacturers.

Exposure Chamber.—The chamber used to expose the contaminated objects was a Scanlan-Morris laboratory autoclave, with an internal volume of about 165 liters. The steam inlet line leading to the chamber was replaced by a pipe which extended out a near-by window. The pipe could be closed at the chamber end by means of a valve. A small vacuum pump was placed in a by-pass in this line so that the autoclave could be evacuated. The condensate return line was used as the entry port, the pipe from the chamber being replaced by a length of copper tubing which led to the cylinder of Carboxide. The cylinder was kept upon a scale so that the weight of gas admitted in each treatment could be determined. The safety valve was not removed from the autoclave, even though it presented a possible source of gas leakage. It is a possibility, when dispensing Carboxide from cylinders, that the low temperature which is reached at the cylinder nozzle may cause the valve to freeze in an open position. It is well, then, to have at least two valves in the line which can withstand the cylinder pressure (1,000 psi), so that the flow into the autoclave may be stopped until the frozen valve can be thawed and shut. While this possibility is being mentioned, it should be added that in these small-scale trials such an occurrence has never been observed.

Exposure Procedure.—The procedure employed was to place the contaminated materials in the autoclave, fasten the door, and evacuate by means of the pump until the gauge indicated a vacuum of about 14 psi. This evacuation served to remove some of the air contained within the interstices of the porous materials being treated, whose presence might otherwise retard somewhat the contact of gas and surface. After about fifteen minutes at this reduced pressure, Carboxide was admitted to the autoclave (the cylinder nozzle was grounded to prevent accumulation of electrostatic charge) until a pressure of 20 to 23 psi gauge was reached. This required between 2 and 3 pounds of gas for the autoclave described.

The materials remained in the sealed autoclave overnight; by morning the pressure had usually dropped by 5 psi. The remaining pressure was released by opening the vent to outdoors; a vacuum was produced in the chamber, and then air was passed through it by means of a small vent valve for about an hour to aerate the materials. At the end of such a treatment, no residual odor of ethylene oxide could be detected in the autoclave or upon the materials exposed therein.

Without a special installation, there is some possibility that the gas mixture issuing from the cylinder will vary in composition as the contents are exhausted. Although we have not performed enough tests, or chemical analyses, to be certain on this score, we have noted no failures in sterilization thus far and believe that under the conditions we describe it need not be a cause of trouble. However, this point will be examined further in some large-scale tests about to be inaugurated.

*Carbide and Carbon Chemicals Corporation, New York, N. Y.

Sampling Procedure for Linens and Blankets.—This study concerned itself primarily with the sterilization of woolen blankets, since this fabric is particularly difficult to treat by the more common procedures without considerable damage. The bacterial population on blankets and other linens was determined by the method described by Lemon.[6] The blanket or sheet was hung over a wire and air was drawn through the fabric for one minute at a rate of 17 liters per minute. This air was passed—by means of a funnel, a length of rubber tubing, and a glass Folin bubbler—through 20 ml. of sterile heart infusion broth. The broth samples were quickly brought to the laboratory, and 1 ml. aliquots were plated both in nutrient and in blood agar and incubated eighteen hours.

After each blanket was sampled, air was blown through the rubber tube and intake funnel to flush out the lint which collected. After a number of items had been sampled in this manner, a one-minute sample of room air was taken to determine whether the room air, or lint which might not have been blown out of the system, might be contributing organisms to the broth collections. Invariably, these final samples contained no bacteria, indicating that substantially all the organisms collected arose from the particular fabric being sampled; the precaution at first adopted, of sterilizing the funnel and tube between samples, was accordingly discarded. (Of course, a separate sterile broth tube and bubbler was used for each blanket.)

The blankets and other linens were sampled in the same way after being removed from the autoclave. With moderate care to avoid excessive handling, no contamination of treated fabrics occurred. As a precautionary measure, some untreated linens were sampled twice in succession in order to be certain that the sampling procedure itself was not removing all the recoverable organisms. In all instances, many organisms were obtained in the second sample.

Other Materials Tested.—In order to obtain data on the susceptibility of certain specific organisms to the action of ethylene oxide, patches of woolen cloth about 1 inch square were inoculated with pure suspensions of two microorganisms. The patches were placed in small Kraft paper envelopes and were steam sterilized before being inoculated. Inoculation was performed by placing two drops of bacterial suspension upon the patch contained in its envelope and then closing the flap. In this way, inoculated and sterile patches could be handled conveniently without contamination. In order to ascertain whether a patch contained viable organisms, it was removed from its envelope by means of sterile forceps and placed in a tube of heart infusion broth, in which it was incubated for eighteen hours at 37° C.

The bacterial suspensions used were a broth culture of *Streptococcus hemolyticus* Group C, and a water suspension of the spores of *Bacillus globigii*. The latter is a nonpathogenic, gram positive, spore-forming rod which grows well on ordinary nutrient media, forming distinctive orange colonies. The resistance of these spores to chemical and physical agents is very great, exceeding that of the anthrax spore in all instances in which the two have been evaluated together.

Tests were performed in which some pages of rather thick volumes (telephone directories, etc.) were contaminated with these organisms, and the books then were placed, both open and tightly shut, in the autoclave.

In one test,* pieces of filter paper were contaminated with a culture of the BCG strain of tubercle bacillus and sampled in a manner analogous to that used for the patches as already described. Some of these papers, as well as some Dubos medium slants containing this organism, were then exposed to Carboxide in the manner indicated.

In all instances, control patches and books were held in the laboratory for as long a time as the test samples were exposed, and then the two sets were plated at the same time. This was done, of course, to determine whether the death of the organisms would have occurred without the treatment.

RESULTS

Tests on Bedding.—Since the purpose of this brief study was not to determine the extent of contamination of hospital equipment, but rather to point out a method of eliminating such contamination should its presence be felt to

*Made possible through the cooperation of Dr. Robert H. Ebert of the Department of Medicine.

constitute a safety hazard, a detailed accounting of the types and numbers of organisms recovered from different materials was not made. However, the colonies obtained from untreated linens in one typical experiment are presented in Table I. Five experiments of this type were performed; every item treated was rendered completely sterile.

An interesting observation concerns the relatively high number of organisms recovered from the soiled pillowcase. This observation may assume special interest in view of some recent reports on the rapid spread of infection in hospital wards from skin lesions.[7]

TABLE I. TREATMENT OF HOSPITAL BEDDING WITH CARBOXIDE

(17 LITERS OF AIR DRAWN THROUGH FABRIC, PASSED THROUGH 20 ML. BROTH; FIGURES REPRESENT NUMBER OF COLONIES OBSERVED FROM A 1 ML. ALIQUOT OF BROTH)

TYPE	COLONIES OBSERVED ON		COLONIES HEMOLYZING BLOOD AGAR
	NUTRIENT AGAR	BLOOD AGAR	
	Before Treatment (as Received)		
Woolen blankets, soiled (Av. of 8)	360	170	5
Sheet, soiled	80	130	1
Pillowcase, soiled	165	180	8
Sheet, clean	60	16	0
Pillowcase, clean	9	7	2
	After Carboxide Treatment		
All materials	0	0	0

In one of the tests, eleven blankets from hospital wards at Great Lakes Naval Training Center, which were used for patients with streptococcal infections, were treated. Hemolytic streptococcus colonies in considerable numbers were noted when samples from untreated blankets were plated in blood agar containing gentian violet. After exposure, all the blankets were completely sterile.

In all, about fifty pieces of bedding were treated in this way, and we observed no instances in which the Carboxide gas failed to produce completely sterile materials.

Tests on Other Contaminated Materials.—Eight test patches containing streptococci and eight containing *B. globigii* spores were exposed in each of five runs, with uniformly sterile results. Patches contaminated but not exposed showed heavy growth in each instance. Fig. 1 illustrates the typical results, the control patch producing innumerable colonies of *B. globigii* while the exposed patch was sterile. That the sterility noted is not due to ethylene oxide carried over into the growth medium is certain for many reasons: the long aerating period removes nearly, if not completely, all the ethylene oxide from the fabric; ethylene oxide is not very effective as a disinfectant in solution until relatively high concentrations are reached; and tests reported elsewhere[2] showed that organisms grew normally even when the sampling liquid or agar was inoculated with amounts of ethylene oxide far exceeding those which might conceivably be introduced along with the bacteria.

Pages of books which were contaminated were completely sterilized by this treatment, even when the book was closed and placed on its side under a considerable amount of other material. There is, incidentally, no observable damage to books, blankets, or sheets, and in tests done elsewhere, analytical balances, spectrophotometers, and other delicate pieces of equipment have been completely sterilized without harm.

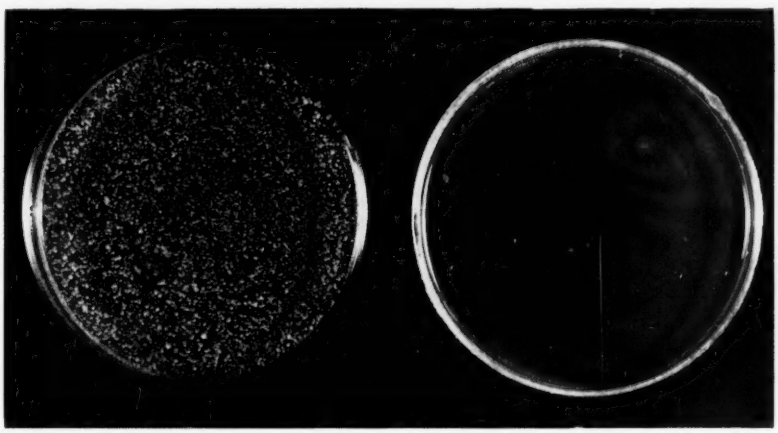

Fig. 1.—Plates poured from broth containing cloth patches inoculated with *B. globigii* spores. Left, untreated patch; right, patch exposed to Carboxide.

The pieces of filter paper containing the BCG organism were completely sterile after treatment. A slant of Dubos medium containing a heavy growth of BCG was exposed, and transplants were found to be incapable of further growth.

The relatively poor sterilizing action of water solutions of ethylene oxide mentioned is indicated by the failure of this method to sterilize completely a water suspension of the BCG organisms (although a great reduction in viable count was observed).

SUMMARY

Since none of the methods currently employed for the sterilization of contaminated hospital equipment are satisfactory, either because they injure certain materials or fail to disinfect others, tests were made of the effectiveness and utility of ethylene oxide in the form of its 10 per cent mixture with carbon dioxide, known as Carboxide.

Hospital blankets and linens, both soiled and laundered, from which it was possible to recover large numbers of microorganisms, were completely sterilized by this treatment. The treatment itself is simple, requiring only an autoclave, a vacuum pump, a cylinder of the gas, and a rudimentary piping system, and is easy and convenient to perform. The expense is moderate and in some cases negligible in view of the results obtained.

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